Cellular Mechanisms of Resveratrol's Interaction with Mitochondrial Reactive Oxygen Species Metabolism

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Abstract

Resveratrol, a polyphenol found naturally in red wines, has attracted great interest in both the scientific community and the general public for its reported ability to protect against many of the diseases facing Western society today. While the purported health effects of resveratrol are well characterized, details of the cellular mechanisms that give rise to these observations are unclear.

Here, the mitochondrial antioxidant enzyme Mn superoxide dismutase (MnSOD) was identified as a proximal target of resveratrol *in vitro* and *in vivo*. MnSOD protein and activity levels increase significantly in cultured cells treated with resveratrol, and in the brain tissue of mice given resveratrol in a high fat diet. Preventing the increase in MnSOD levels eliminates two of resveratrol's more interesting effects in the context of human health: inhibition of proliferative cell growth and cytoprotection. Thus, the induction of MnSOD is a critical step in the molecular mechanism of resveratrol.

Mitochondrial morphology is a malleable property that is capable of impeding cell cycle progression and conferring resistance against stress induced cell death. Using confocal microscopy and a novel 'cell free' fusion assay it was determined that concurrent with changes in MnSOD protein levels, resveratrol treatment leads to a more fused mitochondrial reticulum. This observation may be important to resveratrol's ability to slow proliferative cell growth and confer cytoprotection.

Resveratrol's biological activities, including the ability to increase MnSOD levels, are strikingly similar to what is observed with estrogen treatment. Resveratrol fails to increase MnSOD levels, slow proliferative cell growth and confer cytoprotection in the presence of an estrogen receptor antagonist. Resveratrol's effects can be replicated with

the specific estrogen receptor beta agonist diarylpropionitrile, and are absent in myoblasts lacking estrogen receptor beta. Four compounds that are structurally similar to resveratrol and seven phytoestrogens predicted to bind to estrogen receptor beta were screened for their effects on MnSOD, proliferative growth rates and stress resistance in cultured mammalian cells. Several of these compounds were able to mimic the effects of resveratrol on MnSOD levels, proliferative cell growth and stress resistance *in vitro*.

Thus, I hypothesize that resveratrol interacts with estrogen receptor beta to induce the upregulation of MnSOD, which in turn affects cell cycle progression and stress resistance. These results have important implications for the understanding of RES's biological activities and potential applications to human health.

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Chapter 1. Literature Review

1.1 Introduction

Human health is greatly affected by environmental factors including bioactive molecules found in foods. Plants produce a vast number of diverse secondary metabolites that are being investigated for their potential utility in the prevention and treatment of human disease. Phytoalexins are one such group of molecules that are produced by plants in response to exogenous stressors or pathogen attack, and have potent antimicrobial activity (Ahuja et al., 2012). Interestingly, phytoalexins that occur naturally in edible plants can also inhibit the growth of mammalian cells, including cancers. For example, phytoalexins produced by members of the *Brassica* species (cruciferous vegetables) have an antiproliferative effect on cultured mammalian cells (reviewed in Pedras et al., 2011). Similarly, glyceollins found in soy inhibit the growth of the cancerous prostate cell line LNCaP *in vitro* (Payton-Stewart et al., 2009). The grape vine *Vitis vinifera* produces a number of phytoalexins in response to environmental stress, many of which are abundant in both grapes and red wines (Santamaria et al., 2011). The red wine compound ε-viniferin inhibits the proliferation of vascular smooth muscle cells *in vitro* (Zghonda et al., 2011). Trans-miyabenol C and α-viniferin, two phytoalexins produced by *Vitis vinifera* and found in red wine, inhibit the proliferation of both normal and cancerous human colon cell lines (CCD-18Co, HT-29, HCT-116, Caco-2) *in vitro* (Gonzalez-Sarrias et al., 2011). Thus phytoalexins from a variety of plants exert antiproliferative effects on mammalian cells. In instances where drinks or food are produced from these plants the biologically active phytoalexins are present in the consumed product.

The phytoalexins produced by *Vitis vinifera* and found in red wines became a popular subject of biomedical research following the discovery of the "French Paradox", an observation that the incidence of cardiovascular disease is low in populations in France that regularly consume red wine, in spite of other risk factors including a high fat diet (Renaud and de Lorgeril, 1992). Red wines are a rich source of phytoalexins and polyphenols, containing approximately 2000-6000 mgL^{-1} (Quideau et al., 2011). Of the many phytoalexins found in red wines resveratrol (3,4',5-trihydroxy-*trans*-stilbene; RES) is perhaps the most extensively studied to date. While RES is produced in at least 72 different plant species, some of which are a part of the human diet such as peanuts and mulberries, concentrations of RES are particularly high in red wines (on average 1-3 mgL^{-1}) and they are an important dietary source of this polyphenol (Soleas et al., 1997).

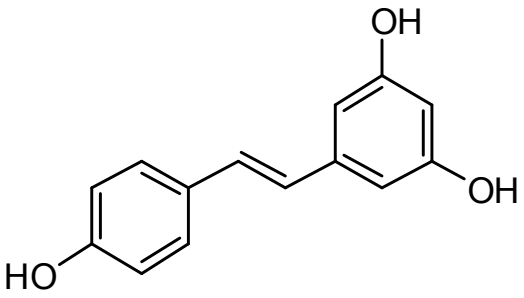


Figure 1.1. Chemical structure of *trans*-resveratrol.

As an isolated compound RES gained attention in both the scientific community and general public for its purported effects on lifespan, cardiovascular disease, diabetes and cancer (reviewed in Smolgia et al., 2012). However, a number of phytoalexins and related compounds in wine and food appear to have similar effects. For example, in the context of RES's anticancer effects pterostilbene, a methylated analogue of RES found in blueberries and wine, can similarly inhibit the proliferation of cancerous cells

(Moon et al., 2012; Mannal et al., 2010). In *Vitis vinifera* RES acts as the parent molecule for a collection of antimicrobial compounds known as viniferins (Soleas et al., 1997), which have recently been reported to possess anticancer properties *in vitro* (Gonzalez-Sarrias et al., 2011). Beyond these closely related structural analogues compounds such as genistein, a polyphenol found in soy products that has a similar phenolic ring structure to RES, inhibits the growth of cancerous cells *in vitro* and *in vivo* (Bielecki et al., 2011; Schleipen et al., 2011). It is clear that RES and other structurally related molecules possess biological activities in mammals. However, the molecular mechanisms responsible are unclear, and the potential for a shared mechanism and in turn a shared therapeutic potential for these molecules has not been fully explored.

1.2 The Health Effects of Resveratrol

Before its well publicized role as a bioactive component of red wines RES was identified as an active ingredient in the Japanese herbal medicine Ko-jon-kon, which was prescribed to treat a wide range of ailments including inflammation, bacterial infections and cardiovascular disorders (reviewed in Soleas et al., 1997). RES is now known for being protective against many of the pathologies facing Western societies today: cancer, neurodegenerative diseases, cardiovascular disease, and type two diabetes (Baur and Sinclair, 2006). There is a considerable amount of experimental data describing these health claims for RES, and this literature review will briefly highlight the best characterized of these.

1.2.1 Anticancer Effects

RES's chemopreventive properties were first described in 1997 by Jang and colleagues in a series of experiments involving both isolated cancer cell lines and mice. Micromolar concentrations of RES effectively inhibited the growth of human promyelocytic leukemia (HL-60) cells *in vitro* (Jang et al., 1997). *In vivo* a topical application of 25 μM RES reduced the number of carcinogen-stimulated skin tumours by 98% in mice (Jang et al., 1997). Following this initial report the anticancer activities of RES have been extended to include a diverse collection of cancerous cell lines. For example, at micromolar concentrations RES inhibits the proliferation of breast cancer cell lines (MCF-7, T47D, NDA-MB-231, LY2, S30) *in vitro* (Damianaki et al., 2000; Bhat et al., 2001). *In vivo* dietary RES supplementation reduces the formation of mammary tumours in Sprague-Dawley rats administered the carcinogen N-methy-N-nitrosurea (Bhat et al., 2001). RES also reduces the proliferative growth of prostate cancer cell lines (LNCaP, PC-3, DU-145) (Hsieh and Wu, 1999), and prevents the development of prostate cancer in a transgenic adenocarcinoma mouse prostate model (Harper et al., 2007). The growth of colon cancer cell lines (CaCo-2, HCA-7, HT-29) is significantly reduced by RES (Schneider et al., 2000; Sale et al., 2004). Thus RES is effective at inhibiting the growth of cancerous cells *in vitro* and in model organisms.

In contrast to RES's well established anticancer properties in cell culture and rodent models, relatively little data is available regarding RES's anticancer effects in humans. However, results from the limited number of existing human trials of RES appear promising. In a recent study patients with colorectal cancer were given an oral resveratrol treatment for eight days. Doses of 0.5g/day and 1.0g/day RES significantly

reduce cell proliferation in cancerous colon tissue (Patel et al., 2010). In a 29 day trial, an oral dose of 2.5g/day RES given to healthy adult subjects significantly decreased plasma levels of insulin-like growth factor 1 and insulin-like growth factor binding protein 3 compared to the placebo control (Brown et al., 2010). This is an important observation given that elevated plasma levels of insulin-like growth factor 1 are associated with increased cancer risk (Renehan et al., 2004; reviewed in Gallagher and LeRoith, 2011). Thus, data from *in vitro* and *in vivo* experiments tentatively support RES's use as an anticancer agent in humans. Further research is required before recommendations for its use can be created, particularly given the estrogenic properties of this molecule (Vang et al., 2011).

1.2.2 Neuroprotection

A second important health claim of RES is its ability to impart neuroprotection. This property has been demonstrated in neuronal cell cultures and also in live animal models of neurodegenerative disease. In cultured cerebellar and dopaminergic neurons treatment with micromolar concentrations of RES significantly reduced cell death caused by exposure to the toxin 1-methyl-4-phenylpyridinium (MPP+), a chemical that interferes with mitochondrial energy production resulting in a model of Parkinson-like neurodegeneration (Alvira et al., 2007; Bournival et al., 2009). Dietary RES supplementation also protects mice (Blanchet et al., 2008) and rats (Jin et al., 2008) against 1-methyl-4-phenyl-1,2,3,3-tetrahydropyridine (MPTP) and 6-hydroxydopamine induced neurodegeneration in experimental models of Parkinson's disease. The protective effect of RES is not limited to the toxic effects of MPP+, as RES treatment similarly protects rat brain hippocampal slices against the damaging effects of oxygen

and glucose deprivation in a model of ischemic brain injury (Zhang et al., 2008). Dietary administration of RES to mice (Dong et al., 2008) or rats (Della-Morte et al., 2009) confers protection against acute brain injury caused by transient middle cerebral artery occlusion or cardiac arrest, respectively. In a transgenic mouse model of Alzheimer's disease dietary RES supplementation reduces plaque formation in brain (Karuppagounder et al., 2009). Thus, long term dietary supplementation with RES can provide neural protection against a variety of potentially cytotoxic stresses and ameliorates neurodegeneration resulting from both acute and chronic insults in rodents.

1.2.3 Cardiovascular Disease and Metabolic Disorders

The application of RES to the prevention and treatment of cardiovascular disease was initiated by an observation that the oxidation of human low-density lipoprotein was inhibited by RES. This was an exciting finding given that the oxidation of low-density lipoprotein is involved in the development and progression of atherosclerosis (Greig et al., 2012). RES protects against myocardial damage following ischemia reperfusion events (Lekli et al., 2008; Monki et al., 2007), and dietary RES supplementation improves heart function in a rat model of hypertension induced heart failure (Rimbaud et al., 2011). Other cardioprotective properties that have been attributed to RES include increased production of nitric oxide (an important signaling molecule in cardiovascular tissues) in cultured human umbilical vein endothelial cells chronically treated with nanomolar concentrations (Takahashi and Nakashima, 2012).

A well publicized health claim of RES is its ability to ameliorate the negative metabolic effects associated with a high fat diet, and to improve insulin sensitivity in models of type 2 diabetes. In adult mice given a high fat, high calorie diet daily RES

enhanced their capacity for endurance exercise and improved their insulin sensitivity (Baur et al., 2006; Lagouge et al., 2006). In high fat diet fed mice receiving 400 mg/kg/day RES supplementation, weight gain associated with the high fat diet was reduced, and the RES fed mice had a significantly lower percentage body fat than the control group provided with the same high fat, high calorie diet (Lagouge et al., 2006). In addition, RES supplementation reduces systemic markers of inflammation and adipogenesis in mice given a high fat, high calorie diet (*e.g*. Kim et al., 2011; Jeon et al., 2012). Overall, in adult male mice RES supplementation is protective against the deleterious effects of a high fat, high calorie diet.

While there have been several studies of RES's effects on high fat diet induced obesity in mice, limited data from human trials exists. In humans, a recent 30 day cross-over study involving a 150mg/day dose of a commercially available form of RES (resVida) improved blood sugar regulation in middle aged obese, but healthy men, although no changes in body weight or composition were observed (Timmers et al., 2011). Similarly, in a study of male patients with type 2 diabetes, four weeks of a twice daily 5mg dose of RES significantly improved insulin sensitivity (Brasnyó et al., 2011). In contrast, 75 mg/day RES for twelve weeks had no significant effect on body composition, mass, energy expenditure or other health paramenters in post-menopausal, non-obese women (Yoshino et al., 2012). Further investigation into the effects of RES supplementation or consumption in food on metabolism and overall health in both males and females is required to appreciate RES's effects in humans.

1.3 Are Resveratrol's Health Effects Unique?

The apparent health effects of RES in rodents have been well explored. Comparatively, little data has been accrued to evaluate the health effects of structurally similar compounds found in wine and other foods. Among the phytoalexins produced in *Vitis vinifera*, pterostilbene and piceid have been recently investigated for their potential anticancer and neuroprotective properties. Pterostilbene inhibits the growth of cancerous breast (MCF7; MDA-MB-231), colon (HCT116, HT29, Caco-2) and prostate cell lines (PC3, LNCaP) (Moon et al., 2012; Mannal et al., 2010; Nutakul et al., 2011; Lin et al., 2009). *In vivo*, dietary pterostilbene imparts protection against cognitive decline in a mouse model of accelerated aging used to approximate the conditions of Alzheimer's disease, and may share RES's well established neuroprotective properties (Chang et al., 2011). Piceid also appears to be neuroprotective, and in rats reduces oxidative stress in brain tissue, and rescues learning deficits following an ischemic event (Li et al., 2012). It therefore seems that at least two of RES's important health effects, anticancer activities and neuroprotection, are shared by other structurally similar compounds found in red wines.

1.4 Molecular Mechanisms of Resveratrol in Mammals

Much effort has been put forth to define the health effects of RES, but the downstream targets and signaling pathways that give rise to these effects continue to be a subject of much debate within the scientific community. RES appears to influence the activity of a vast number of enzymes, and the list of downstream targets and affected signaling pathways is continually growing, making an evaluation of essential target

molecules a challenge. A common theme in the ailments that are positively affected by RES is an increase in oxidative stress.

Indeed, many of RES's observed effects are consistent with a reduction in intracellular oxidative stress, *i.e.* a reduction in the oxidation of low-density lipoprotein, and an increased resistance to oxidant induced cell death (Frankel et al., 1993; Jin et al., 2009). RES does have inherent antioxidant activity related to its chemical structure, but is a fairly weak chemical antioxidant and radical scavenging would likely be observed only at supraphysiological concentrations that are above 0.1M (Hu et al., 2007; Leonard et al., 2003). A more plausible hypothesis is that RES reduces oxidative stress by modulating the activity of endogenous antioxidant enzymes.

1.4.1 Oxidative Stress and Antioxidant Enzymes

Animal cells are continuously exposed to reactive oxygen and nitrogen species (referred to collectively here as ROS) that are generated as a consequence of aerobic respiration. In many cell types endogenously produced ROS originate in the mitochondria from a one electron reduction of molecular oxygen to produce the superoxide anion ($O_2^{\cdot -}$) (reviewed in Turrens 2003). Superoxide production occurs at complex I and complex III of the mitochondrial electron transport chain, resulting in the presence of superoxide in the matrix and mitochondrial intermembrane space. Superoxide itself is not highly reactive with intracellular macromolecules (reviewed by Benov, 2001), but can be converted to more potent oxidizing agents. Superoxide reacts to produce hydrogen peroxide through both enzymatic and non-enzymatic reactions, and the hydrogen peroxide produced may undergo Fenton chemistry in the presence of free transition metals to produce the very highly reactive hydoxyl

radical (·OH). Superoxide may also react with nitric oxide (NO) in an uncatalyzed reaction to form the strong oxidant peroxynitrite (ONOO-) (Pryor and Squadrito, 1998). It is hypothesized that the production of superoxide accounts for less than 1% of the total mitochondrial oxygen consumption (St. Pierre et al., 2002). However, the damaging effects of unmitigated superoxide production are clearly demonstrated by the necessity of Manganese superoxide dismutase (MnSOD) in mammals, a mitochondrial enzyme whose sole enzymatic activity is the dismutation of superoxide to produce water and hydrogen peroxide (Li et a., 1995; Lebovitz et al., 1996).

Intracellular levels of ROS are affected by both their rate of production and their rate of detoxification by intracellular antioxidant enzymes. Damage to proteins, lipids and DNA occurs when ROS production overwhelms the capacity of the antioxidant systems that maintain their intracellular concentrations under normal conditions. Oxidation of amino acid residues in proteins may negatively affect their structure and in turn their function. Heavily oxidized proteins can impede the activity of the proteasomal machinery required for protein homesotasis leading to cell death (reviewed by Davies, 2001). Lipid peroxidation can negatively affect membrane fluidity and the activity of membrane bound proteins, again leading to cell death (Chen and Yu, 1994). Oxidative damage to DNA is perhaps the most deleterious consequence of oxidative stress, and can increase genomic instability leading to elevated incidence of cancer and can prevent necessary transcription and replication events (reviewed in Finkel and Holbrook, 2000). The accumulation of oxidative damage over time has been hypothesized to underlie the aging process (Harman 1956; Harman 1972, but see also Pérez et al., 2009 for a critical review) and the development of some age-

associated diseases such as Parkinson's (reviewed in Danielson and Andersen, 2008). Yet in addition to their potentially harmful role in the cell, ROS may act as signaling molecules, and play an important role in the regulation of the cell cycle (reviewed by Menon and Goswami, 2007). Modulation of intracellular ROS levels through the actions of antioxidant enzymes is therefore important in regulating cell functions including proliferation.

Superoxide produced in the mitochondria is converted to hydrogen peroxide and water in a reaction catalyzed by Mn superoxide dismutase (MnSOD) in the mitochondrial matrix and CuZn superoxide dismutase in the cytosol and intermembrane space of mitochondria (as shown in equation 1.1, Okado-Matsumoto and Fridovich, 2001). Hydrogen peroxide may be further detoxified to produce water by the enzymes glutathione peroxidase and catalase. An upregulation of antioxidant enzymes, including MnSOD, occurs in response to treatment with mild oxidative stressors (Mates et al., 1999). Antioxidant enzymes are important determinants of intracellular ROS levels and in turn play an important role in preventing oxidative damage and mediating the signaling functions of ROS.

Equation 1.1: Superoxide anion conversion to hydrogen peroxide catalyzed by superoxide dismutase.

$$M^{(n+1)+}SOD + O_2\cdot^- \rightarrow M^{+}SOD + O_2$$
$$M^{+}SOD + O_2 + 2H^{+} \rightarrow M^{(n+1)+}SOD + H_2O_2$$

(M) represents the transition metal

1.4.2 Induction of Antioxidant Enzymes by Resveratrol

RES treatment confers protection against exogenous oxidative stressors, and the production of ROS is reduced in RES treated cells (Movahed et al., 2012; Albani et al.,

2009; Lu et al., 2008; Ungvari et al., 2009). RES's ability to reduce oxidative stress, and enhance cellular stress resistance is consistent with an induction of cellular antioxidant enzymes. Similar to what is observed with RES treatment, overexpression of MnSOD, CuZn superoxide dismutase or glutathione peroxidase in mammalian cells can confer an increased ability to withstand oxidative stressors including hydrogen peroxide and the superoxide generator paraquat (St. Clair et al., 1991; Chen et al., 2000; Faucher et al., 2003). In human lung fibroblasts (MRC5) treated with micromolar concentrations of RES for 48h or two weeks a significant increase in MnSOD protein levels and activity is observed, an effect that reaches nearly 5 fold at the two week time point (Robb et al., 2008a). The increase in MnSOD in this cell type is accompanied by a slight increase in the activity of glutathione peroxidase, but is not accompanied by a general increase in antioxidant enzyme activity, as CuZn superoxide dismutase and catalase activity are unaffected by RES (Robb et al., 2008a). The observation that RES increases MnSOD protein levels and activity was subsequently repeated in skeletal muscle, neuronal cell lines and in human coronary endothelial cells (Ryan et al., 2010; Fukui et al., 2010; Kairisalo et al., 2010; Ungvari et al., 2009). Thus, an increase in MnSOD elicited by RES is a robust observation that has been made in a variety of cell types.

MnSOD is similarly upregulated by the activation of multiple signaling pathways that influence animal health and lifespan. For example, MnSOD is a downstream target of the transcription factor FOXO3a which is regulated by the insulin/insulin-like growth factor-1 pathway, a highly conserved signaling pathway shown to influence both cellular stress resistance and lifespan in a variety of animal models (Kops et al., 2002,

Baba et al., 2005; Yamamoto et al., 2005; reviewed by Longo and Fabrizio, 2002). MnSOD is induced in response to the pharmacological inhibitor of mTOR rapamycin, which extends lifespan in mice (Iglesiase-Bartolome et al., 2012; Harrison et al., 2009). Furthermore, MnSOD is upregulated by activation of the transcriptional coactivator Peroxisome Proliferator-activated Receptor Gamma Coactivator 1-alpha, which is involved in the cellular response to mild oxidative stress (St-Pierre et al., 2006) and in response to physical activity (Handschin and Spiegelman, 2008). Thus MnSOD is an important downstream target of several interventions linked to animal health.

The increase in MnSOD that occurs following RES treatment is particularly interesting in light of the importance of MnSOD in cell physiology.
MnSOD homozygous null mice exhibit a severe phenotype of metabolic acidosis, fatty liver and severe dilated cardiomyopathy, and die within 10 to 18 days after birth (Li et al., 1995; Lebovitz et al., 1996). MnSOD heterozygous null mice survive into adulthood, but have an increased incidence of cancer (Van Remmen et al., 2003), and a heightened sensitivity to oxidative stress (Tsan et al., 1998; Kim et al., 2002). On a cellular level MnSOD levels are closely tied to rates of cell proliferation. Cancerous cell lines often have low levels of MnSOD that are accompanied by rapid rates of cell division. Transgenic overexpression of MnSOD into cancerous cell lines slows proliferative growth (Li et al., 1998; Ough et al., 2004; Venkataraman et al., 2005).

In addition to its effects on cell division, MnSOD also influences cellular stress resistance. MnSOD overexpression in pheochromocytoma cells (PC6) protects against apoptosis induced by excess iron, the β-amyloid peptide and nitric oxide producing compounds (Keller et al., 1998). Mouse mesenchymal cells (C3H10t1/2) that

overexpress MnSOD are protected from cell death induced by the toxin paraquat (St. Clair et al., 1991). It is clear that MnSOD influences many important aspects of cell function, and it is likely that its induction is an important biological activity of RES. However, the observation that MnSOD levels increase following RES treatment only supports a correlative role for this enzyme, and does not address the hypothesis that the induction of MnSOD is an essential mechanistic step in RES's cellular effects.

1.4.3 The Activation of Sirtuins as a Molecular Mechanism of Resveratrol

The biological activities of RES have been hypothesized to arise from its interaction with numerous substrates. One of the most pervasive hypotheses that has been put forward to account for RES's biological activities is an activation of sirtuin 1 (SIRT1), a deacetylase enzyme with a broad range of molecular targets. In yeast, Sir2 was identified as a putative longevity protein following the observation that its overexpression extends lifespan in *Caenorhabditis elegans* (Tissenbaum and Guarente, 2001, but see Burnett et al., 2011). Mammalian SIRT1 is closely related to the *C. elegans* protein Sir2 and RES was identified as a potent activator of SIRT1, increasing its reported catalytic activity by approximately 13-fold over control (Howitz et al., 2003). However, the evidence to support a direct SIRT1 activation by RES is at best equivocal, with strong experimental evidence to dispute this claim.

The initial experiments that indentified RES as a direct activator of SIRT1 were based on a deacetylation assay that relied on a fluor-de-lys reporter system (Howtiz et al., 2003). It was later discovered that the fluor-de-lys fluorophore interacts directly with RES resulting in an artificially high signal. RES does not increase the deacetylase activity of SIRT1 when given its native substrate in the absence of the

fluorophore. Data from assays involving the fluor-de-lys fluorophore are therefore confounded by the artifactually high signal generated in the presence of RES (Pacholec et al., 2010; Beher et al., 2009; Borra et al., 2005; Kaeberlein et al., 2005). Thus the observation of a direct activation of SIRT1 that occurs through an allosteric interaction with RES is not a robust result.

There is limited support for a direct interaction with sirtuins as a mechanistic basis of RES's biological activities; however, it is clear that sirtuins do have essential roles in many aspects of cell physiology and are therefore likely to be tangentially involved in RES's effects. For example, acetylation and deacetylation regulate important intracellular processes, including various aspects of the cellular stress response (*i.e.* FOXO transcription factors, heat shock protein factor 1), and cell metabolism (*i.e.* PGC1-alpha) (Brunet et al., 2004; Westerheide et al., 2009; Nemoto et al., 2005). Deletion of SIRT1 in mammals is extremely harmful, resulting in metabolic dysregulation and increased incidence of autoimmune disease (Seifert et al., 2012; Sequeira et al., 2008). SIRT1 activity is increased by RES treatment in many different experimental contexts (reviewed in Baur 2010), but the severe negative consequences of SIRT1 deletion make elucidating its role in the mechanism of RES a challenge. Given the importance of sirtuins in biology and the growing list of proteins whose functions are modified by acetylation, it is likely that the increase in SIRT1 activity that is observed with RES treatment plays an important, but not directive, role in RES's molecular mechanism.

1.4.4 Resveratrol as a Phytoestrogen

Nearly a decade before RES was proclaimed a direct activator of SIRT1 it was identified as an estrogen receptor (ER) agonist (Gehm et al., 1997). Interestingly there is remarkable overlap between the health effects of RES and those of estrogen. Similar to RES estrogen is neuroprotective, and prevents neurodegeneration in models of Parkinson's disease, Alzheimer's disease and ischemia reperfusion (reviewed in Wise et al., 2002; Brinton 2008). RES and estrogen both have beneficial effects on metabolism. Estrogen has regulatory effects on energy homeostasis and body composition (reviewed in Faulds et al., 2012). In ovariectomized rats that are hyperphagic and have increased adiposity compared to age matched controls, estrogen supplementation effectively prevents the obeseogenic effects of a high fat diet (Stubbins et al., 2011). Cardioprotection is another shared property of RES and estrogen. Depletion of estrogen via ovariectomy increases the susceptibility of female mice to ischemia-reperfusion damage, and this can be effectively reversed by estrogen treatment (Nikolic et al 2007).

On a mechanistic level, RES is structurally similar to the synthetic estrogen diethylstilbestrol, is capable of binding to ERs and stimulates a transcriptional response (Gehm et al., 1997). RES stimulates the transcription of an estrogen controlled luciferase reporter gene in a dose dependent manner a human breast cancer cell line (MCF-7). Estrogen antagonists inhibit the activation of the reporter gene by RES, and in a competition assay RES successfully prevented the binding of radiolabeled estradiol to ERs, demonstrating that RES is an estrogen receptor agonist *in vitro* (Ghem et al., 1997).

In other experimental contexts RES has been described as a Selective Estrogen-receptor Modulator (SERM), which is term used to describe a group of estrogens capable of eliciting both agonist and antagonist effects on the ERs. As would be predicted for an SERM, RES's effect on proliferation is dependent on the cell type. While RES stimulates proliferation in an osteoblastic cell line (MC3T3-E1), it inhibits proliferation in some estrogen dependent breast cancer cell lines (MCF-7) and in colon cancer cell lines (HT-29) (Mizutani et al., 1998; Kim et al., 2004; Juan et al., 2008). RES elicits an estrogenic response in the estrogen-sensitive pituitary cell line PR1; however, in contrast to other known estrogens, it does not stimulate growth (Stahl et al., 1998). Dietary intake of RES fails to mimic the actions of estrogen on reproductive tissues and growth in weanling rats (Turner et al., 1999). Although RES does not dramatically affect reproductive physiology many of its *in vivo* effects, such as its positive effects on bone health in ovariectomized rodents, are very similar to observations made with estrogen treatment (Liu et al., 2005). The discrepancies in RES's ER agonist activity and the differences in effect between cell types may be attributed to its ability to bind both ERalpha and ERbeta.

1.4.5 Estrogen Receptors Alpha and Beta

RES is an agonist for both ERalpha and the more recently discovered ERbeta (Bowers et al., 2000). ERalpha is the predominant estrogen receptor in female reproductive physiology. In contrast, ERbeta is the predominant ER in many tissues including brain and colon in males and females (reviewed in Zhao et al., 2010). The function of ERbeta in these varied tissues is currently unclear. Data gathered using ERbeta agonists suggests that activation of this receptor is antiproliferative in dividing

cells (Ström et al., 2004; Hartman et al., 2009). The ERbeta agonist diarylpropionitrile (DPN) inhibits the growth of the murine colon cancer cell line MC38 in vitro, and of cells in the colon and small intestine of ovariectomized rats (Motylewska et al., 2009; Schleipen et al., 2011).

Early studies of RES's actions as an estrogen agonist did not differentiate between its binding to ERalpha or ERbeta, and did not consider how the relative proportion of each receptor varied between cell types. In fact, RES was discounted as an ER agonist by some researchers due to its failure to elicit dramatic effects on reproductive physiology (For example see Kondratyuk et al., 2011). A re-evaluation of the importance of ER binding to RES's effects is necessary, particularly in light of the increasing appreciation for the cellular functions of ERbeta. Furthermore the role of estrogen signaling in the activities of other red wine polyphenols has not yet been well characterized. There are many polyphenols found in red wines and other foods that are predicted to bind to ERs. A recent computational study screened 51 plant extracts for their ability to bind ERalpha and ERbeta, and identified over a dozen compounds including RES that bind preferentially to ERbeta over ERalpha (Yuan et al., 2011). The cellular effects of these compounds have not been described in detail, and the potential overlap in RES's activities and shared molecular mechanisms has not yet been explored.

1.5 Bioavailability of Resveratrol

RES is renowned for its *in vitro* effects, but its therapeutic applications may be limited by its poor bioavailability (Baur and Sinclair, 2006). RES undergoes extensive chemical modification in the intestinal tract and is rapidly metabolized leading to low

concentrations and a very short half life of approximately 0.6h in plasma. In humans, a 25mg oral dose gives an average peak concentration of 2μM RES in plasma (Walle et al., 2004). In rodents an oral dose of RES in the hundreds of milligram range only yields plasma and tissue levels in the low micromolar range, with the highest concentrations being observed in liver and kidney tissue (Teng et al., 2012; Marier et al., 2002; Juan et al., 2010). RES is capable of crossing the blood brain barrier, and the unmodified compound has been detected in brain tissues of rats and mice given oral doses of RES (Juan et al., 2010; Vitrac et al., 2003). Chronic intake of RES has been explored as a potential strategy to circumvent RES's low plasma and tissue levels but appears to be ineffective, as repeated bolus dosing of RES does not increase its accumulation in extravascular tissues (Asensi et al., 2002; Almeida et al., 2009).

The low bioavailability of RES raises questions as to how this compound alone is able to elicit physiological effects following dietary intake. Interestingly, while levels of the pure compound are very low, there is agreement between the biological activities observed *in vitro* using supraphysiological concentrations (micromolar range), and those observed following dietary intake. Asensi et al., (2002) provided experimental evidence that the antiproliferative effects of RES in a cultured B16 melanoma cell line could be replicated by dietary supplementation. To evaluate whether a similar inhibition of growth could be observed *in vivo*, B16 melanoma cells were injected into the fat pads of control and RES fed mice. The B16 melanoma cell injection resulted in tumour development at the injection site and in liver tissue in the control group. The group given RES in their diet was protected from the hepatic invasion of the cancerous cells that was observed in the control group, supporting the idea that RES can inhibit the growth of the

same cell line *in vitro* and *in vivo* (Asensi et al., 2002). A potential explanation for the apparent discrepancy between *in vitro* and *in vivo* concentrations may be that the more abundant RES metabolites are capable of eliciting cellular effects similar to RES; however, this idea has not yet been investigated. Thus, while RES does have very low bioavailability its *in vivo* activities nonetheless do reflect what is observed *in vitro* and dietary intake may be an effective anticancer strategy.

1.5.1 Resveratrol Delivery and the Need for a High Fat Diet

Understanding the factors that influence bioavailability following RES delivery is an important research goal as it can aid in creating dietary strategies to maximize uptake. An appreciation for dietary RES delivery is particularly important, as it is found naturally in foods and in some instances is being added to foods and beverages at a supplement level (*i.e.* Gaudette and Pickering, 2011). In rodent models the effects of dietary RES are observed only when provided as an additive in a high fat diet. For example, statistically significant effects on insulin sensitivity and aerobic exercise endurance are observed only when RES is added to diets with fat contents equal to or greater than 40%, and there are no significant effects on the measured parameters when RES is added to a standard composition diet (Baur et al., 2006; Lagouge et al., 2006). The basis of the necessity of a high fat diet in RES's dietary effects is unclear. One possibility may be that a high fat diet changes the absorption and metabolism of RES, which is a lipophillic compound. RES undergoes extensive chemical modification in the intestinal tract, and its inclusion in a high fat diet may alter the degree to which it is modified (Kuhnle et al., 2000).

To fully appreciate the potential effects of RES, delivery methods that can increase its concentrations in all tissues and allow for lower, more easily achieved doses

are necessary. Alternative methods of shielding RES from intestinal modifications have been reported including encapsulation in a nanoparticle delivery system, liposome encapsulation and the synthesis of more bioavailable derivatives (see Teskac and Kristl, 2010; Gokce et al., 2012; Coimbra et al., 2011), but their ability to increase RES's bioavailability has not been thoroughly tested *in vivo* (Teskac and Kristl, 2010; Gokce et al., 2012).

1.6 Hypotheses and Proposed Approach

The biological activities of RES are consistent with a change in reactive oxygen species metabolism. In agreement with this idea an induction of the mitochondrial antioxidant enzyme MnSOD occurs with RES treatment in a variety of cell types (Robb et al., 2008a; Ryan et al., 2010; Fukui et al., 2010; Kairisalo et al., 2010; Ungvari et al., 2009). Overexpression of MnSOD alone gives rise to many of the same observations as RES treatment, including reduced rates of cell proliferation and increased resistance to oxidative stress (Li et al., 1998; Ough et al., 2004; Venkataraman et al., 2005; St. Clair et al., 1991; Keller et al., 1998). It is plausible that MnSOD induction is an essential step in RES's molecular mechanism; however direct evidence in support of this hypothesis has not yet been provided.

To elucidate the role of reactive oxygen species metabolism in RES's molecular mechanisms cell proliferation, stress resistance, and the activities or protein levels of the antioxidant enzymes catalase, glutathione peroxidase, MnSOD and CuZn superoxide dismutase were measured in control and RES treated human lung fibroblasts (MRC5; selected as a representative non-cardiovascular, noncancerous cell line), human neuroblastomas (SHSY-5Y; selected as a neuronal cell line) and mouse myoblasts (C2C12; selected as a noncancerous, highly metabolic cell line). RES's effects on proliferation and stress resistance in the absence of MnSOD induction were measured to clarify the role of this enzyme in RES's molecular mechanism. If MnSOD is indeed a critical target of RES, it is hypothesized that inhibiting its induction will prevent the changes in proliferative cell growth and cytoprotection that are associated with RES treatment.

The upstream signaling pathways that are responsible for RES's activities are controversial. Two ideas that have been put forward to account for RES's effects were explored: SIRT1 activation and RES's actions as an ER agonist. In the context of SIRT1 involvement, the sirtuin inhibitor sirtinol and a SIRT1 null embryonic fibroblast cell line were used to determine what role, if any, SIRT1 had on RES's ability to upregulate MnSOD. In regards to determining the importance of RES's actions as an ER binding compound, ER agonists, antagonists and ERbeta null myoblast cell lines were used. If ERs are necessary for RES's ability to upregulate MnSOD it is hypothesized that pharmacological inhibition of their activity would prevent RES's effects. Similarly it is anticipated that RES will not affect proliferation, stress resistance or MnSOD in cell lines that do not express ERs.

Mitochondria are an important site of RES's biological activities. Mitochondrial morphology can influence bioenergetics, cellular stress resistance and proliferation in a manner that is consistent with observations made for RES treatment. The ability of RES to influence the morphology of the mitochondrial reticulum has not been explored. In this thesis the influence of RES on mitochondrial morphology in cultured cells was evaluated using confocal microscopy and a cell free fusion assay that measures mitochondrial fusion. It is predicted that RES will affect mitochondrial morphology leading to a highly fused mitochondrial reticulum.

While RES is certainly the most well studied phytoalexin in wine, many dietary compounds appear to have similar benefits to human health. It is currently unknown whether other phytoalexins found in red wine can exert effects similar to RES in mammalian cells through a common molecular mechanism. To address the hypothesis

that RES's biological activities are shared between other structurally related dietary molecules the effects of four compounds with structures closely related to RES and seven phytoestrogens were tested for their effects on proliferative cell growth, stress resistance and the levels of intracellular antioxidant enzymes. It is hypothesized that if these compounds do share RES's molecular mechanism their cellular effects and key target enzymes, including MnSOD, will be similar.

RES elicits a change in the levels of intracellular antioxidant enzymes, and in particular MnSOD levels, in a variety of cell types *in vitro*. It is unknown whether RES affects the protein level and activity of antioxidant enzymes *in vivo*. To address this question antioxidant enzyme protein levels and/or activity levels were measured in the brain, heart, liver and kidney tissues of mice given RES in a high fat, standard diet or via a subcutaneous osmotic minipump. Two novel silicon formulations were also used to deliver RES at supplemental levels. It is hypothesized that RES will increase the protein level and activity of MnSOD *in vivo*.

Overall, it is hypothesized that the induction of MnSOD elicited by RES *in vitro* is a key step in its ability to reduce proliferative cell growth and increase stress resistance, and that this effect will exist *in vivo*.

Chapter 2. MnSOD is an Essential Target of Resveratrol that is Required for its Effects on Cell Proliferation and Stress Resistance

Hypothesis

An induction of MnSOD is required for RES to inhibit proliferative cell growth and confer cytoprotection. The induction of MnSOD occurs via estrogen receptor signaling, and is independent of SirT1.

Objectives

The objectives of this project were to: **1)** Determine if the induction of MnSOD is a requirement for RES's effects on cell proliferation and stress resistance, and **2)** Evaluate the importance of SirT1 and estrogen receptors in the RES-induced upregulation of MnSOD.

Publication of results

Robb EL, Stuart JA. Resveratrol interacts with estrogen receptorβ to inhibit cell replicative growth and enhance stress resistance by upregulating mitochondrial superoxide dismutase. Free Radical Biology and Medicine. 50: 821-831, 2011.

Contributions

I performed all experiments, statistical analysis and manuscript preparation.

2.1 Introduction

RES, a plant polyphenol that is bioactive in animals, has attracted research interest due to its reported benefits to human health. Micromolar concentrations of RES have been shown to enhance the stress resistance of a wide range of cultured mammalian cell lines (reviewed in Robb and Stuart, 2010). Dietary RES supplementation has been associated with improved cardiovascular performance and resistance to acute neural trauma and neurodegeneration (Baur and Sinclair, 2006). Given the potential significance of these findings, the development of a detailed understanding of the mechanisms underlying these RES effects is of considerable interest.

RES was previously identified for its ability to extend the lifespan of yeast, fruit flies, nematode worms and fish (Baur and Sinclair, 2006). This effect was tied to a stimulation of SirT1, a protein deacetylase, by an allosteric interaction of the enzyme with RES (Howitz et al., 2003; Wood et al., 2004). However, there have been difficulties confirming some of these longevity data (Bass et al., 2007; Kaeberlein et al., 2005). Similarly, the assay used to demonstrate RES's stimulation of SirT1 may generate a misleading artifactual signal (Kaeberlein et al., 2005; Borra et al., 2005; Pacholec et al., 2010). It is thus unclear whether SirT1 or other members of the sirtuin family directly interact with RES to give rise to its effects, and it remains to conclusively identify the mechanisms of RES's actions in animal cells and tissues.

Recently, the mitochondrial antioxidant enzyme Mn-superoxide dismutase (MnSOD) was identified as a downstream target of RES in human cells (Robb et al., 2008a). Similar observations have been made with other dietary polyphenols with similar structural attributes (Borrás et al., 2006). Dietary delivery of RES was also able

to induce a significant upregulation of MnSOD in mouse brain (Robb et al., 2008b; Chapter 6). These results are particularly interesting given the similarity of effects between MnSOD overexpression and RES treatment. For example, MnSOD transgenic mice are resistant to acute cerebral injuries (reviewed in Robb and Stuart, 2010), and MnSOD overexpressing mitochondria are resistant to permeability transition (Silva et al., 2005). MnSOD overexpression also slows mitotic growth of a variety of mammalian cells both *in vitro* (e.g. Sarsour et al., 2008) and *in vivo* (Kim et al., 2010), which is strikingly similar to the S phase delay elicited by micromolar concentrations of RES (e.g. Hu et al., 2007; Zhou et al., 2009). Taken together, these results suggest that the ability of RES to modulate MnSOD expression underlies some of its most important biological activities.

Here, an RNAi knockdown of MnSOD is used in diverse mammalian cell types to demonstrate that both the increase in cellular stress resistance and the delayed replicative growth elicited in mammalian cells by micromolar RES are mediated by MnSOD induction. RES was classified as a phytoestrogen (Gehm et al., 1997) prior to identification as a putative activator of sirtuins. Therefore, the role of estrogen receptors in the RES-induced MnSOD upregulation was explored using inhibitors and agonists of estrogen receptors alpha and beta. These data indicate that RES interacts with ERbeta to increase MnSOD expression in different cell lines, rendering these cells slow growing and resistant to a variety of exogenous stressors.

2.2 Experimental Procedures

2.2.1 Materials:

Modified Eagle Medium with Earle's salts, l-glutamine and sodium bicarbonate, Dulbecco's Modified Eagle Medium with high glucose, l-glutamine and sodium bicarbonate and Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 were obtained from Sigma–Aldrich (St. Louis, MO). Penicillin/streptomycin, non-essential amino acids, and fetal bovine serum were obtained from Hyclone (Logan, UT). RES was obtained from A.G. Scientific (San Diego, CA). Sirtinol and Diarylpropionitrile (DPN) were obtained from Tocris Bioscience (Ellisville, MO). Propylpyrazole triol (PPT) was obtained from Cayman Chemicals (Ann Arbor, MI). Cytotoxicity Detection Kit^{TM} was purchased from Roche Applied Science (Laval, Canada). Bradford BioRad Protein Assay dye was purchased from BioRad laboratories (Hercules,CA). Amplex UltraRed was purchased from Invitrogen (Burlington, Canada). Prestained broad range protein marker was purchased from New England BioLabs (New England, MA). Pierce Memcode Reversible Protein Stain Kit^{TM} was obtained from Thermo Fisher Scientific (Mississauga, Canada). MnSOD and CuZnSOD antibodies were purchased from Stressgen (Victoria, Canada). Actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxiredoxin 1 and peroxiredoxin 3 antibodies were purchased from Abcam (Cambridge, MA) Infrared dye-conjugated secondary antibodies to rabbit and mouse were purchased from Rockland Immunochemicals (Gilbertsville, PA). siRNA to MnSOD, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a scrambled control sequence, and NeoFx Transfection Agent^{TM} were purchased from Ambion (Austin, TX). All other chemicals and purified enzymes were obtained either from Sigma–Aldrich (St.

Louis, MO), BioShop (Burlington, Canada) or Fisher Scientific (Mississauga, Canada) unless otherwise stated.

2.2.2 Cell Lines and Culture Conditions

MRC5 (Coriell), C2C12 (Sigma) and SHSY5Y (ATCC) cell lines were cultured in accordance with the distributor's protocol. $SirT1^{-/-}$ and $SirT1^{+/+}$ MEFs were a generous gift from Dr. Michael McBurney at the University of Ottawa. They were generated as described in (McBurney et al., 2003). All cell lines were cultured under conditions of 37 °C, humidified to 5% CO_2, 18% O_2 atmosphere, with the exception of the $SirT1^{-/-}$ and $SirT1^{+/+}$ MEFs which were cultured at 3% O_2. Cell density was determined by counting using trypan blue exclusion. Solutions of RES (Final concentration 10-50μM), sirtinol (Final concentration 60μM), estradiol (Final concentration 1nM-10μM), ICI 182780 (Final concentration 1nM-10μM), PPT (Final concentration 1nM-10μM) and DPN (Final concentration 1nM-10μM) were added directly to culture media and refreshed daily.

2.2.3 siRNA Treatment

MRC5 and C2C12 cell lines were transfected with NeoFx Transfection Agent™ and 2-10nM siRNA to MnSOD, GAPDH or a scrambled control sequence at a density of $1.5x10^5$ cells/mL, and $1.0x10^6$ cells/mL, respectively. siRNA containing medium was replaced 24h after transfection. Experiments in the presence of siRNA were performed 24-72h following transfection. Reduction in MnSOD protein levels was determined via Western blot.

2.2.4 Stress Resistance and Death Experiments

To evaluate stress resistance, cells were washed three times with PBS and incubated with serum free medium containing 2% BSA with or without the following chemical stressors: Paraquat (1.5-3.5mM; 6hr), Hydrogen Peroxide (60-120µM; 2h), and MMS (0-1.8 mM; 24h). Following the incubation time the stressor containing media was removed, cells were rinsed three times with PBS and incubated with serum containing medium for up to 18h. Cell number was calculated at various time points following the stressor exposure via trypan blue exclusion and cell counting. Samples of media were taken at various time points for LDH release measurements.

2.2.5 Lactate Dehydrogenase Activity

Lactate dehydrogenase (LDH) activity was measured in a solution containing 20mM HEPES buffer (pH 7.3), 0.2mM NADH and 200µL culture medium as in Vallés et al., 2008. LDH activity was followed spectrophotometrically by the rate conversion of NADH to NAD^+ at 340nm. In RNAi experiments, LDH release was measured using the Cytotoxicity Detection Kit™, per the manufacturer's instructions, as this kit is more accurate at the very low volumes used in these experiments.

2.2.6 Preparation of Whole Cell Lysates

Cells were lysed by incubation for one hour in ice cold lysis buffer (10mM Tris pH 8.0, 150mM NaCl, 2mM EDTA, 2mM Dithiothreitol, 0.4mM PMSF, 40% (v/v) glycerol, 0.5% (v/v) NP40 with periodic sonication (Ultrasonic Inc., Sonicator W-375; setting 3). After incubation, cells were centrifuged at 10 000 g (4˚C) for 10 minutes (Thermo Scientific, IEC Micromax/Micromax RF). The protein concentration of the resulting

supernatant was determined by the Bradford method using a BioRad protein determination kit. Whole cell lysates were stored at -80˚C.

2.2.7 Western Blots

Equal amounts of whole cell lysate (10μg) were separated by SDS-PAGE (5% stacking, 12% resolving gels) and electroblotted onto a polyvinylidene fluoride membrane. Memcode Reversible Protein Stain Kit™ was used in accordance with the manufacturer's instructions to verify equal protein loading/transfer. Following blocking, membranes were incubated with antibodies to MnSOD (1:5000 dilution) or CuZnSOD (1:2500 dilution) or peroxiredoxin 1 (1:1000) or peroxiredoxin 3 (1:1000). The membranes were visualized using the Odyssey infrared imaging system from LI-COR Biosciences, with IR-linked secondary antibodies (1:5000 dilution). Western blot analysis was performed using Odyssey imaging software 1.0.

2.2.8 Hydrogen Peroxide Detection

To determine if RES affected cellular hydrogen peroxide production the rate of its accumulation in PBS was measured. Exponentially growing MRC5 cells were collected by trypsinization and washed 4 times in PBS. Cells were resuspended in PBS and incubated at 37˚C with gentle agitation. The rate of hydrogen peroxide accumulation in PBS was monitored for up to 2 h. During the 2h incubation no change in cell viability was observed. Hydrogen peroxide was detected using Amplex UltraRed reagent, following the manufacturer's instructions. The addition of 10 Units/mL catalase (CAT) was used to confirm that the observed changes in fluorescence were due to the presence

of hydrogen peroxide. A standard curve of hydrogen peroxide concentrations was established using PBS collected from a cell suspension at time 0.

2.2.9 Glutathione Peroxidase Activity

Glutathione peroxidase activity was measured in a solution containing 50mM potassium phosphate buffer (pH 7.0), 0.4mM EDTA, 0.15mM β-NADPH, 1 unit glutathione reductase, 1mM glutathione and 10μg of protein. The addition of 0.007% H_2O_2 was used to initiate the reaction, and the change in absorbance was measured at 340nm.

2.2.10 Citrate Synthase Activity

Citrate synthase activity was measured in a buffer containing 50 mM Tris pH 8.0, 0.5 mM 5,5'-dithiobis(2-nitrobenzoic acid), 0.1 mM acetyl-coenzyeme A, 0.05% Triton X-100 and 10 μg protein. The reaction was initiated by the addition of 0.5 mM oxaloacetate, and absorbance was followed at 412nm.

2.2.11 Statistical Analysis:

Data were analyzed by repeated measures ANOVA using Systat v.12. Post-hoc comparisons between means were analyzes by Tukey's test. Data comparing two experimental groups were analyzed using the student's t-test. All data are presented as means ± standard error of the mean (SEM). A p-value of < 0.05 was considered significant.

2.3 Results:

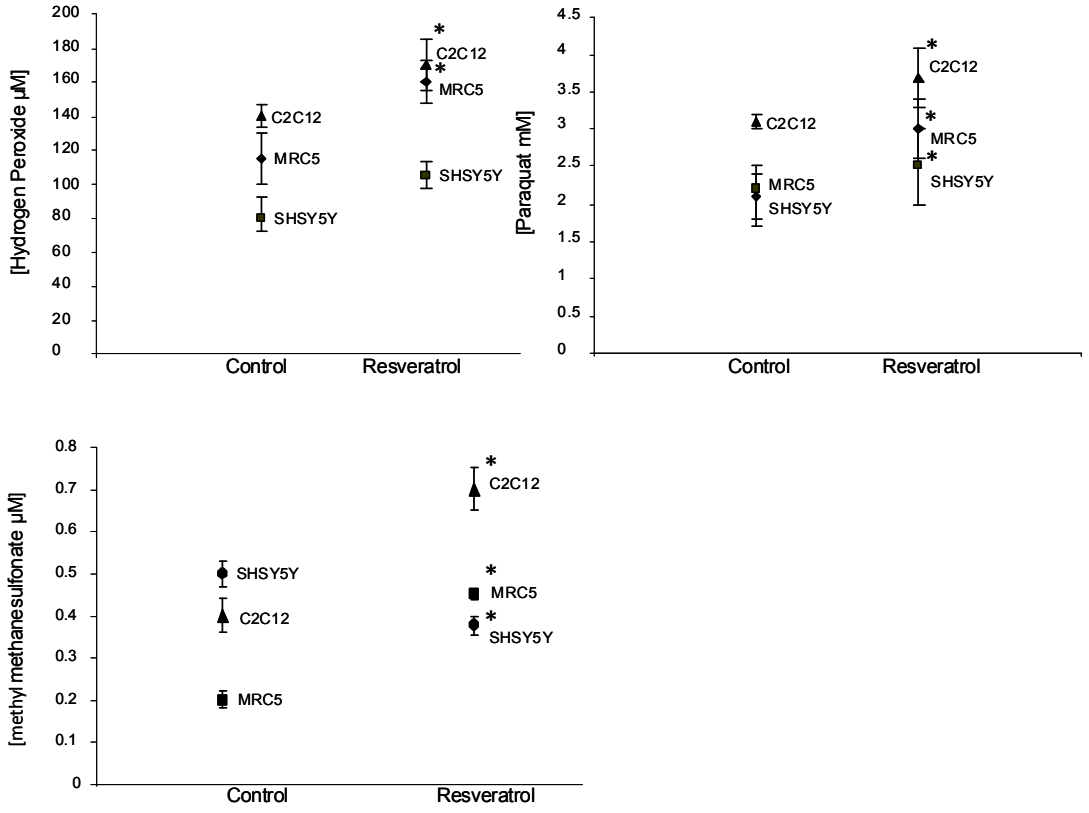
Many of RES's reported effects are consistent with an increase in cellular stress resistance (Robb and Stuart, 2010). To investigate long term effects of RES on stress resistance three mammalian cell lines were incubated with RES for 48h (which was removed prior to exposure to the stressors) and were subsequently exposed to: hydrogen peroxide and paraquat, and the methylating agent methyl methanesulfonate (MMS). Pre-treatment with RES significantly increased resistance to hydrogen peroxide and paraquat in all three cell lines. Similarly, a significant increase in resistance to MMS was observed in the MRC5 and C2C12 cell lines (Fig 2.1A).

An increase in the protein level or activity of the mitochondrial SOD, MnSOD, was concurrent with the RES mediated increase in stress resistance (Fig 2.1B). RES has been reported to increase mitochondrial density (Robb et al., 2008a). Therefore, to determine whether the observed increase in MnSOD was simply due to increased mitochondrial number, MnSOD protein level measurements were normalized to the activity of citrate synthase, a marker of cellular mitochondrial content. A slight increase in citrate synthase activity with RES was observed; however, the increase in MnSOD protein level remained when mitochondrial density was accounted for. Increased activity of MnSOD is predicted to increase rates of hydrogen peroxide production (Buettner et al., 2006). A subtle, but statistically significant increase in the activity of glutathione peroxidase was observed in RES treated cells (Fig 2.1C).

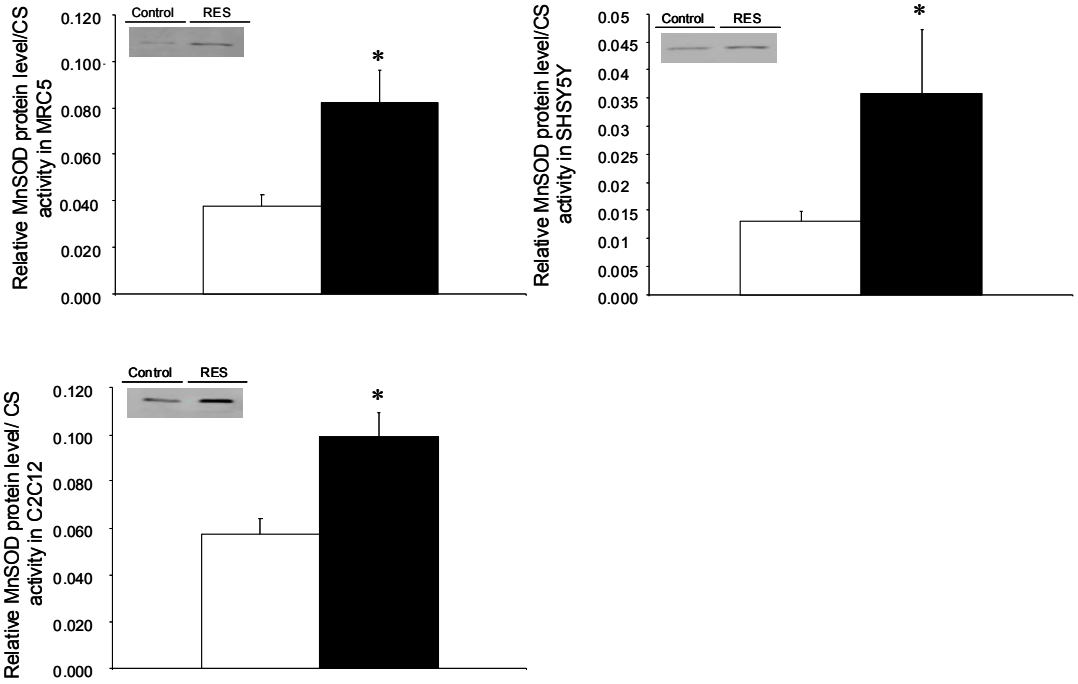
To further examine whether increases in MnSOD protein levels were accompanied by increased production of hydrogen peroxide in the mitochondria or cytosol, the protein levels of peroxiredoxin 1 and 3 protein were assessed using the

method described by Cox et al., 2010. Peroxiredoxins are thiol peroxidases that scavenge hydrogen peroxide, and form intermolecular disulfide bonds during their catalytic cycle to produce a dimer. Capture of the reduced monomer by methylation, and quantification of the ratio of the dimer to monomer form by immunoblotting can be used to assess the intracellular redox environment. Neither peroxiredoxin 1 nor peroxiredoxin 3 showed a significant change in the presence of RES (Fig 2.1D). Similarly, there was no significant difference between hydrogen peroxide production in control and RES treated cells (Fig 2.1E). No changes in CuZn superoxide dismutase or catalase activities were observed with RES (data not shown).

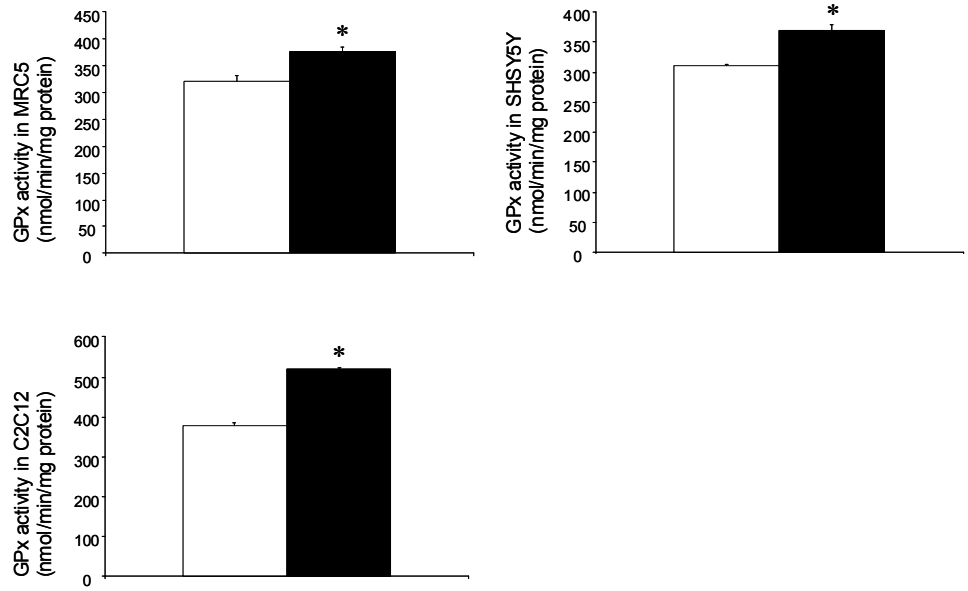
2.1 A.



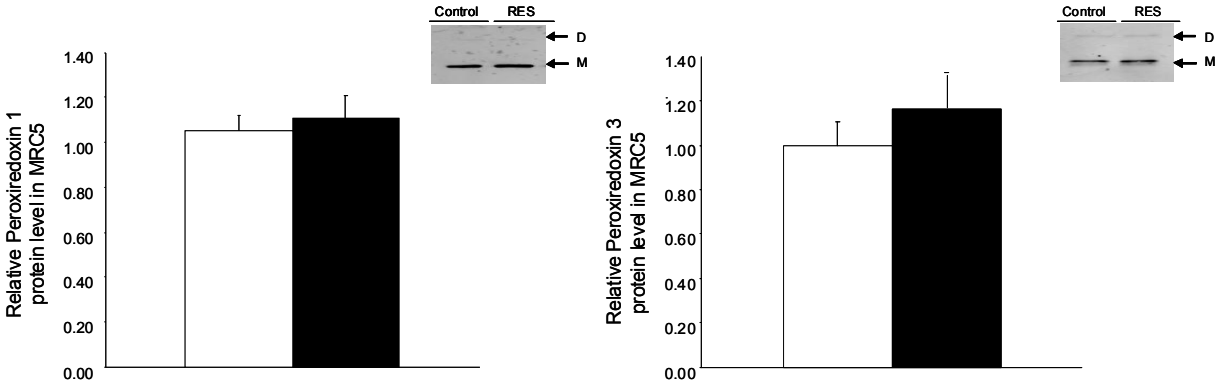
2.1 B.


Control
RES
Relative MnSOD protein level/CS activity in MRC5
0.120
0.100
0.080
0.060
0.040
0.020
0.000
*
Relative MnSOD protein level/CS activity in SHSY5Y
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0.000
Relative MnSOD protein level/ CS activity in C2C12

2.1 C.


GPx activity in MRC5 (nmol/min/mg protein)
450
400
350
300
250
200
150
100
50
0
*
GPx activity in SHSY5Y (nmol/min/mg protein)
GPx activity in C2C12 (nmol/min/mg protein)
600
500

2.1 D.



2.1 E.

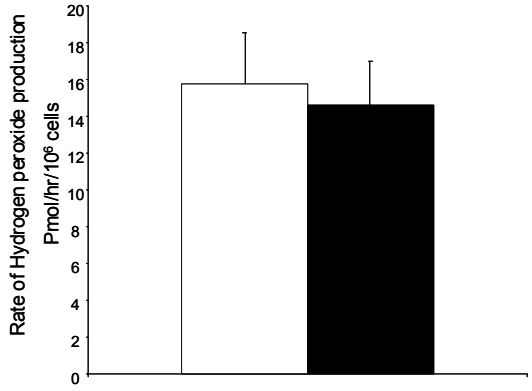


Figure 2.1. Resveratrol increases cellular stress resistance and intracellular antioxidant enzymes.
A. LD50 values for hydrogen peroxide, paraquat or methyl methanesulfonate for MRC5, SHSY5Y or C2C12 cell lines pre-treated with RES for 48h. Cell death evaluated by LDH release. Data shown in A represent the means of measurements from 5-6 independent trials. Error bars represent SEM. *=$p<0.05$. B. Relative MnSOD protein standardized to citrate synthase activity in MRC5, SHSY5Y or C2C12 cell lines treated with DMSO (vehicle control) or RES for 48h. C. Glutathione peroxidase activity in MRC5, SHSY5Y or C2C12 cell lines treated with control or RES for 48h. D. Relative peroxiredoxin 1 and peroxiredoxin 3 protein levels in MRC5 treated with DMSO (vehicle control) or RES for 48h. E. Hydrogen peroxide production in MRC5 treated with DMSO (vehicle control) or RES for 48h. Unfilled bars represent vehicle control. Filled bars represent 50μM RES. Data shown in B and C represent the measn of duplicate measures from 3 independent trials. Error bars represent SEM. *=$p<0.05$.

A second important biological activity for RES is its reported ability to slow replicative growth. MnSOD has been previously shown to influence mitotic growth (e.g. Zhou et al., 2008). Population doubling time and MnSOD levels in C2C12 and MRC5 cells were followed over a one week of incubation with RES. RES treatment increased population doubling time, and this was concomitant with an increase in MnSOD protein levels (Fig 2.2).

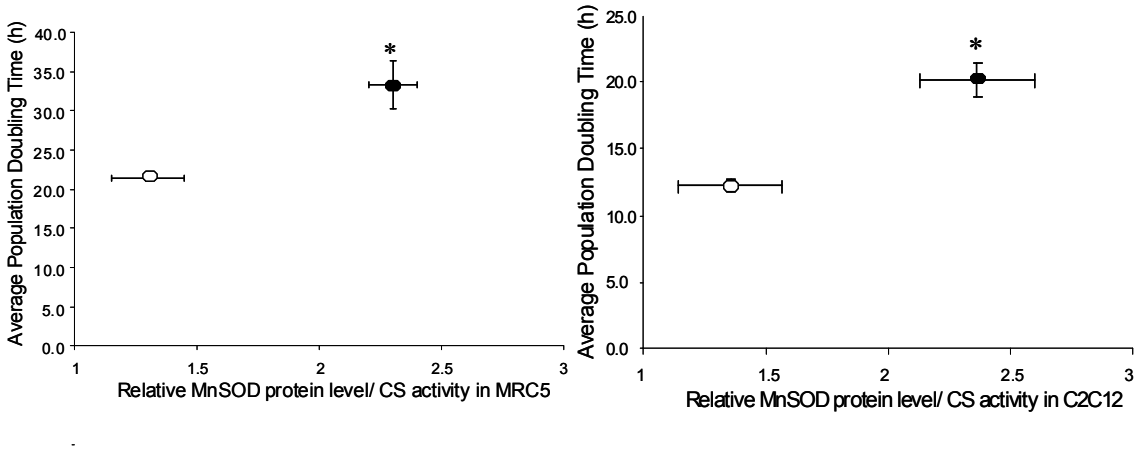


Figure 2.2. Resveratrol induced increase in MnSOD is concurrent with a decrease in cell proliferation.
Average population doubling time vs. average MnSOD protein level in MRC5 or C2C12 myoblasts treated with DMSO (vehicle control) or RES for one week. Unfilled circles represent vehicle control. Filled circles represent 50μM RES. Data shown represents the means of 9 independent trials. Error bars represent SEM. *=$p<0.05$.

To assess the role of MnSOD in the increased cellular resistance and slowed replicative growth observed in the presence of RES, siRNA was used to inhibit the RES stimulated induction of MnSOD. MnSOD siRNA reduced MnSOD protein levels in control cells and prevented the RES-induced increase in MnSOD protein (Fig. 2.3A; MRC5 cells shown; a similar effect observed in C2C12 is not shown). In the absence of a MnSOD induction, RES had no significant effect on replicative growth rate (Fig 2.3B), nor did it render any of the three cell lines more resistant to hydrogen peroxide or

paraquat (Fig 2.3C; MRC5 cells shown; similar effects observed in C2C12 and SHSY5Y, not shown). Neither the GAPDH siRNA nor the scrambled siRNA controls affected either replicative growth rates or stress resistance.

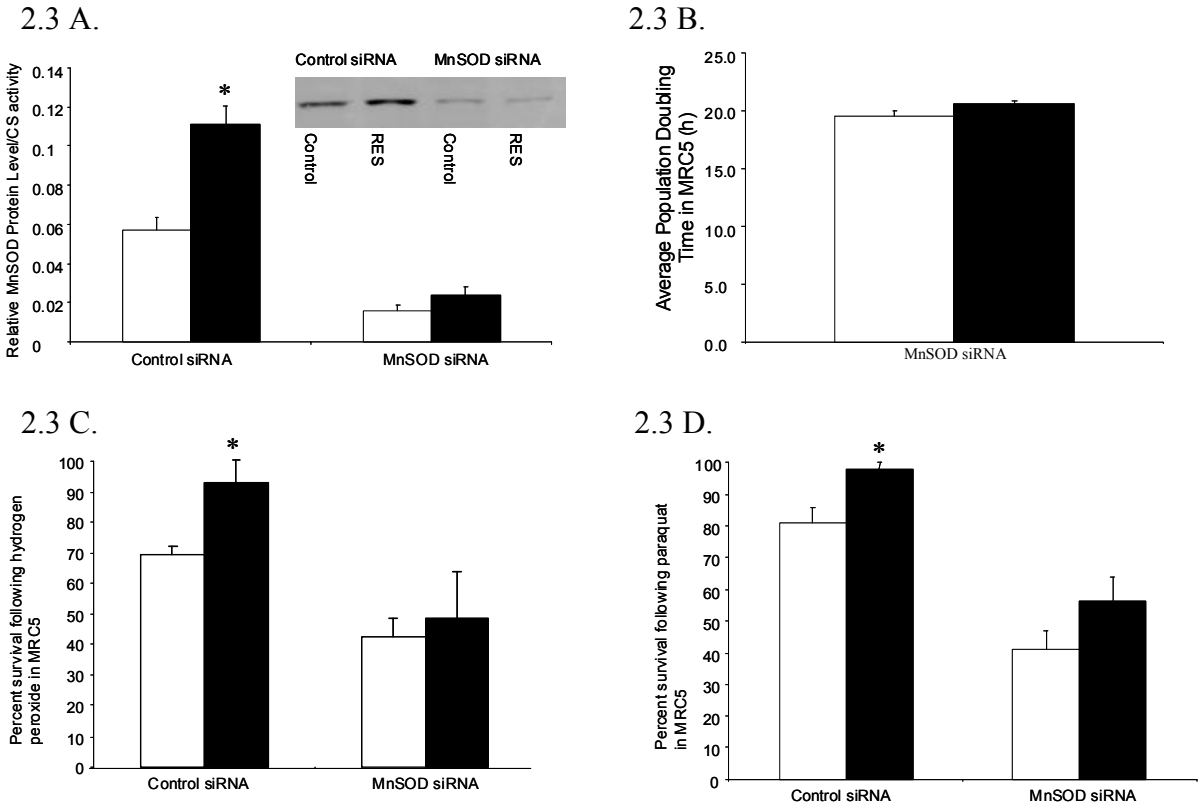
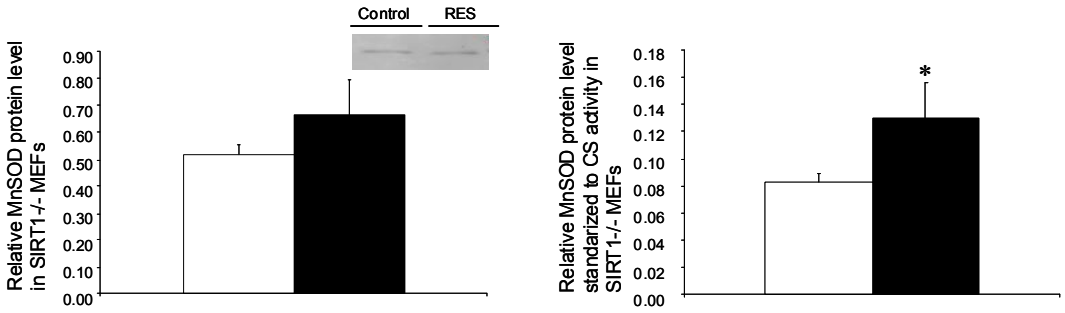


Figure 2.3. MnSOD is essential for resveratrol's effect on cellular stress resistance and population doubling time.
A. Relative MnSOD protein level in MRC5 lines treated with control siRNA or MnSOD siRNA ± 50μM RES. B. Effect of 50μM RES for 72h on average population doubling time in MRC5 cells treated with MnSOD siRNA. C. Percent survival in MRC5 cells 6h following exposure to hydrogen peroxide (70μM, 2h), and 3h following exposure to paraquat (1mM, 24h). Cell lines were treated with control or MnSOD siRNA ±50μM RES for 48h prior to stress exposure. Unfilled bars represent vehicle control. Filled bars represent 50μM RES. Data shown represent the means of 3 independent trials. Error bars represent SEM. *=$p<0.05$.

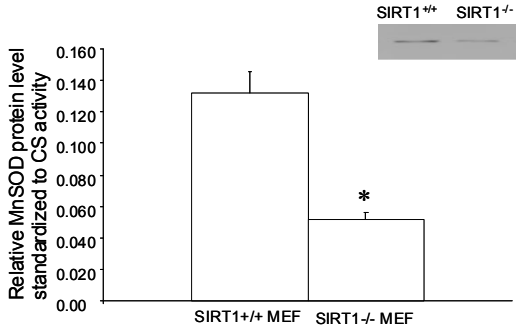
Many of RES's biological activities have been attributed to a stimulation of SirT1 activity. To determine whether RES's effect on MnSOD is mediated by SirT1, SirT1$^{-/-}$ mouse embryonic fibroblasts (MEF) were used. Under control conditions, SirT1$^{-/-}$ MEFs

expressed substantially lower levels of MnSOD and had a lower mitochondrial content (citrate synthase activity) than control SirT1$^{+/+}$ MEFs (Fig 2.4B), perhaps suggesting that a complete deletion of SirT1 generally affects mitochondrial biology, including the expression of mitochondrial antioxidant enzymes. Nonetheless, RES treatment induced an increase in MnSOD protein level in SirT1$^{-/-}$ cells when differences in mitochondrial abundance were taken into consideration (Fig 2.4A). To further evaluate the role of SirT1 in the RES mediated induction of MnSOD, MRC5 and C2C12 cells were treated with Sirtinol, an inhibitor of SirT1 activity. The induction of MnSOD with RES treatment persisted in the presence of Sirtinol, although this increase was slightly lower in magnitude (Fig 2.4C). Together, these data support the hypothesis that mechanisms beyond SirT1 are involved in RES's effect on MnSOD levels.

2.4 A.



2.4 B.



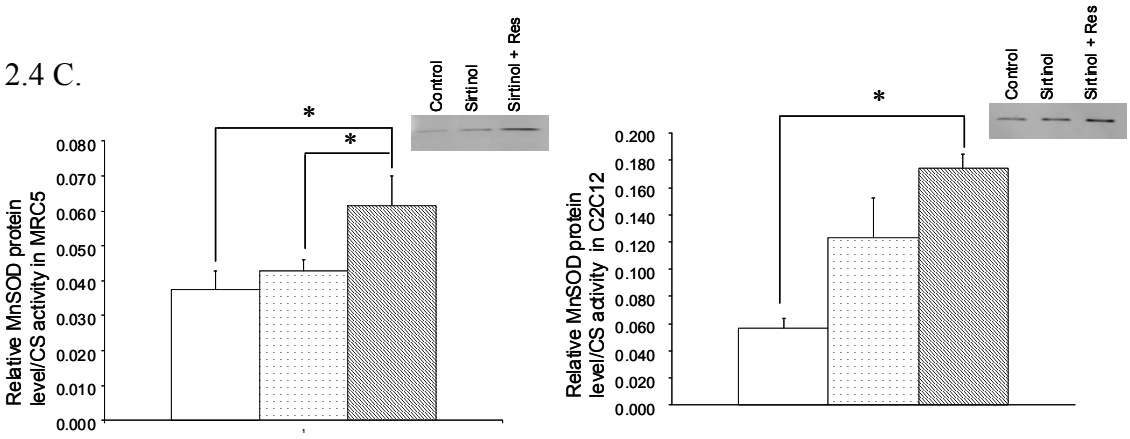
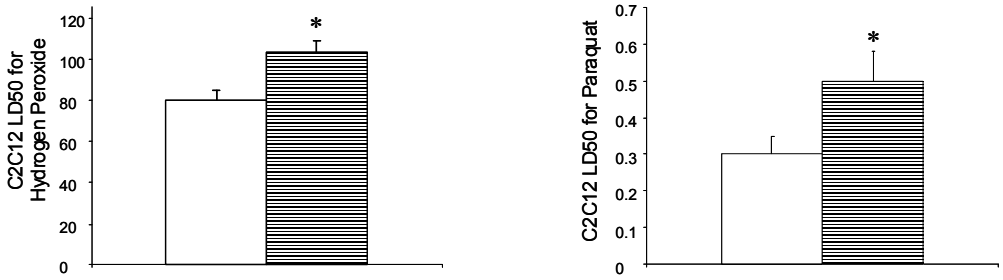


Figure 2.4. Resveratrol increases MnSOD independently of SIRT1.
A. Resveratrol increases MnSOD independently of SIRT1. B. Relative MnSOD protein level standardized to citrate synthase activity in SIRT1+/+ and SIRT1-/- MEF's not treated with RES. C. MRC5 and C2C12 cell lines treated with DMSO (vehicle control) or sirtinol ± RES ± 50μM RES for 48h. Unfilled bars represent vehicle control. Dotted bars represent 60μM sirtinol. Diagonal line bars represent 60μM sirtinol + 50μM RES. Data shown represents the mean of duplicate measures from 3 independent trials. Error bars represent SEM. *=$p<0.05$.

RES has been previously identified as a phytoestrogen (Gehm et al., 1997). As a first step, the effect of estradiol on stress resistance and antioxidant enzymes was evaluated. Following the same treatment regime used for RES, it was observed that incubation with 1μM 17β-estradiol significantly increased resistance to hydrogen peroxide and paraquat (Fig 2.5A), while also increasing MnSOD protein level and glutathione peroxidase activity (Fig 2.5B). An estrogen receptor antagonist, ICI 182780 was used to validate that the estradiol effect was due to estrogen signaling. To evaluate the possibility that the RES's effect on MnSOD may be related to its ability to act as an estrogen receptor agonist, the inhibitor ICI 182780 was used in conjunction with RES and measured resistance to hydrogen peroxide or paraquat, and MnSOD protein levels. Interestingly, in the presence of ICI 182780 RES's ability to protect against hydrogen

peroxide and paraquat was significantly reduced (Fig 2.5C). The induction of MnSOD following RES treatment was also significantly reduced in the presence of ICI 182780 (Fig 2.5D), supporting the hypothesis that RES's effect on MnSOD is mediated through its interactions with estrogen receptors.

2.5 A.



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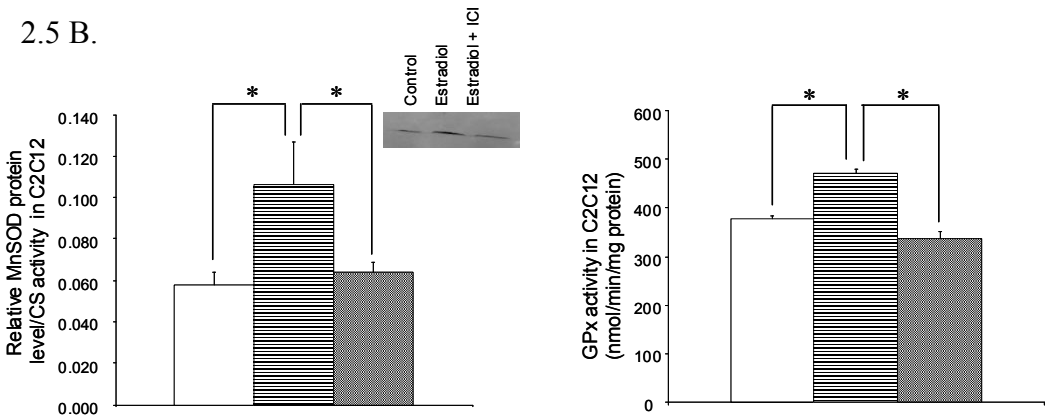

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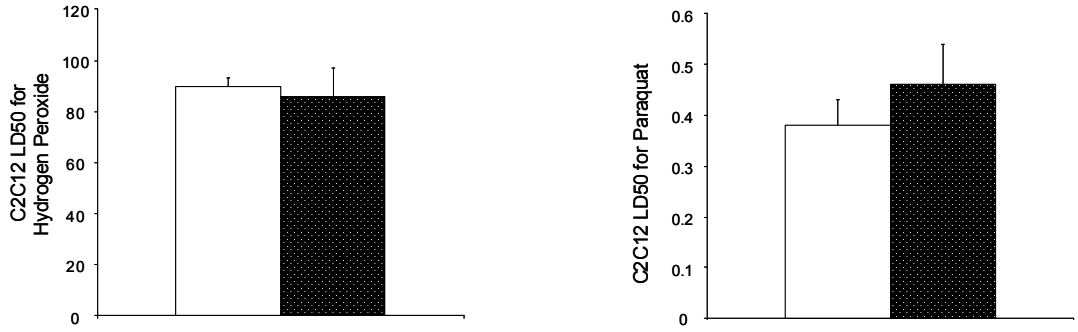


2.5 D.

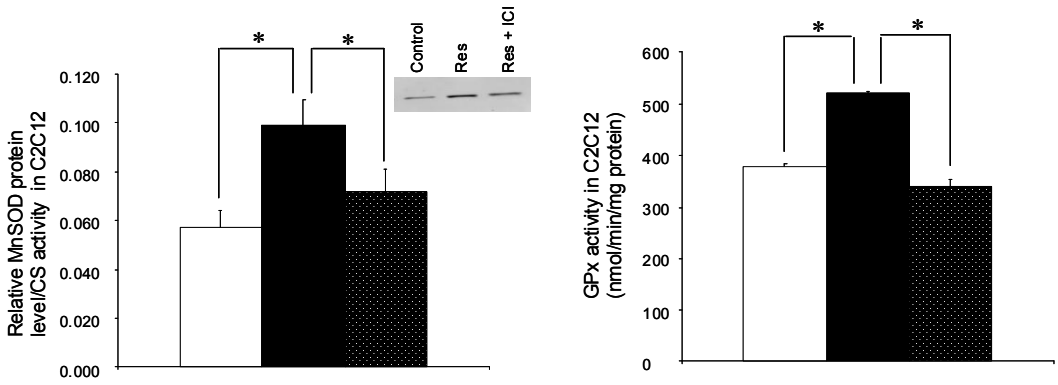


Figure 2.5. Resveratrol's effects may be related to its actions as a phytoestrogen. A. LD50 values for hydrogen peroxide or paraquat for C2C12 myoblasts pre-treated with DMSO (vehicle control) or 1μM β-estradiol for 48h. Cell death evaluated by LDH release. Data shown represents the mean of measures of 5-6 independent trials. Error bars represent SEM. *=$p<0.05$. B. Relative MnSOD protein level standardized to citrate synthase activity. Glutathione peroxidase activity in C2C12 myoblasts treated with control or 1μM β-estradiol ± ICI 182780 for 48h. C. LD50 values for hydrogen peroxide, paraquat for C2C12 myoblasts pre-treated with control or RES ±ICI 182780 for 48h. Cell death evaluated by LDH release. Data shown represents the mean of measurements from 5-6 independent trials. Error bars represent SEM. *=$p<0.05$. D. Relative MnSOD protein level standardized to citrate synthase activity, glutathione peroxidase activity in C2C12 myoblasts treated with control or RES ± ICI 182780 for 48h. Unfilled bars represent vehicle control. Horizontal lines represent 1μM β-estradiol. Shading represents 1μM β-estradiol + 1μM ICI 182780. Filled bars represent 50μM RES. White dots represent 50μM RES ± 1μM ICI 182780. Data shown represents the mean of duplicate measures of 3 independent trials. Error bars represent SEM. *=$p<0.05$.

There are two predominant estrogen receptor isoforms: ERalpha and ERbeta (Cheskis et al., 2007). To further explore the role of these receptors in MnSOD expression, specific agonists to ERalpha and ERbeta were used. Treatment with the ERbeta agonist DPN significantly increased MnSOD protein level, while this effect was absent in cell lines treated with the ERalpha agonist PPT (Fig 2.6).

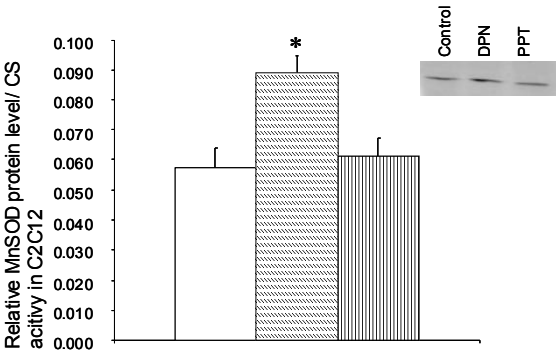


Figure 2.6. Estrogen receptor beta agonist DPN increases MnSOD protein level . Relative MnSOD protein level standardized to citrate synthase activity in C2C12 cell line treated with vehicle, DPN or PPT. Unfilled bars represent vehicle control. Bars with hatch marks represent 1μM DPN. Bars with vertical lines represent 1μM PPT. Data shown represent the means of duplicate measures of 3 independent trials. Error bars represent SEM. *=$p<0.05$.

2.4 Discussion:

Two of RES's most exciting effects in the context of human health are its ability to confer cytoprotection and to slow rates of proliferative cell growth. These effects are concurrent with, and dependent upon, the ability of RES to increase levels of MnSOD. This observation is particularly interesting given the importance of MnSOD. In many cell types, mitochondria are the primary producer of intracellular reactive oxygen species (ROS), and also harbour the redox sensitive signaling proteins necessary for apoptosis (Turrens et al., 2003; Kagan et al., 2009). MnSOD, the sole SOD of the mitochondrial matrix, catalyzes a critical first step in the detoxification of ROS produced by the electron transport chain. *In vivo*, deletion of the MnSOD gene in mice greatly reduces lifespan, as the mice suffer from severe cardiomyopathy and neurodegeneration (Li et al., 1995; Lebovitz et al., 1996). Hearts of MnSOD haploinsufficient mice are more susceptible to apoptosis induced by oxidative stress (Loch et al., 2009). In contrast, manipulation of MnSOD expression is protective against stress-induced cell death in a variety of experimental contexts (Robb and Stuart, 2010). Overexpression of MnSOD can protect against neuron death in ischemia and also in murine models of neurodegeneration such as Alzheimer's disease (Keller et al., 1998; Dumont et al., 2009). While ROS contribute to macromolecule damage and can have negative consequences in cell function, they are hypothesized to act as important signaling molecules. Mitochondrial superoxide is thought to act as a mitogen, and its conversion to hydrogen peroxide by MnSOD may act as a signal to slow cell replication (Sarsour et al., 2008). In MEFs progression through the cell cycle is influenced by MnSOD activity. Increased MnSOD activity promotes transition into quiescence, whereas MEFs deficient in MnSOD cannot exit the cell cycle

(Sarsour et al., 2008). Similarly, overexpression of MnSOD in transformed cell lines is associated with reduced cellular replication rate (Li et al., 1998; Weydert et al., 2006). Thus, MnSOD has been established as an important enzyme in stress resistance and cell proliferation both *in vitro* and *in vivo*. The ability of RES to increase MnSOD protein levels is critical to two of its biological effects, suggesting that MnSOD is an essential component in RES's mechanism of action.

Buettner et al. (2006) predict that increasing MnSOD activity on the order of three fold may lead to a two fold increase in intracellular concentrations of hydrogen peroxide, a product of superoxide dismutation. In contrast, it has also been hypothesized that in a system where the interaction between superoxide and nitric oxide is minimal, SOD intercepts approximately 90% of superoxide, and therefore increasing concentrations of SOD in the absence of an increase in superoxide will have minimal effects of the production of hydrogen peroxide (Liochev and Fridovich 2007). Production of hydrogen peroxide by mitochondria under basal conditions is extremely low, making reliable measurements difficult (St-Pierre et al., 2002). Therefore, changes in hydrogen peroxide production were monitored by measuring enzymes involved in its detoxification and its diffusion from intact cells. A subtle increase in the activity of glutathione peroxidase was observed in cells treated with RES and estradiol. Glutathione peroxidase is responsible for the reduction of hydrogen peroxide, and exists as multiple isoforms within the cell. The assay used here measures the activity of glutathione peroxidase 1, a cytosolic and mitochondrial isoform found predominately in the cytosol. Given the relatively low abundance of mitochondria in cultured cells and the high proportion of glutathione

peroxidase 1 found in the cytosol, it is unlikely that the observed increase in glutathione peroxidase activity is exclusively mitochondrial.

The peroxiredoxin family of enzymes has an important role in maintaining redox balance, and the presence of specific isoforms localized to different intracellular compartments presents a means to monitor redox changes in specific organelles (Cox et al., 2010). The oxidized form of peroxiredoxin 1 (cytosolic), or peroxiredoxin 3 (mitochondrial) were not observed at quantifiable levels in control or RES treated cells, suggesting that the increase in MnSOD and glutathione peroxidase observed with RES did not promote an oxidizing intracellular environment. Furthermore, there was no significant difference in hydrogen peroxide production between control and RES treated cells. These data demonstrate that the RES-induced increase in MnSOD does not substantially increase intracellular concentrations of hydrogen peroxide.
This observation is in agreement with Fukui et al., (2010), who observed a decrease in intracellular ROS concentrations in RES treated mouse hippocampal neurons concomitant with an increase in MnSOD activity. It is plausible that low level, localized changes in hydrogen peroxide imparted by an increase in MnSOD may serve a signaling function, and its interaction with redox sensitive transcription factors, such as NFκB may mediate some of RES's effects. Alternately, MnSOD may participate in direct interactions with cellular proteins to exert its effects. For example, MnSOD has been reported to bind to, and stabilize p53 (Lui et al., 2008; Behrend 2005). Further research into the effect of RES on redox sensitive processes is required.

Understanding the various pathways upstream of MnSOD induction is critical to RES's further therapeutic development. Recently, there have been contradicting data in

the literature surrounding the allosteric activation of the sirtuin family of proteins by RES (Howitz et al., 2003; Wood et al., 2004; Kaeberlein et al., 2005; Borra et al., 2005; Pacholec et al., 2010). It was observed that RES's effect on MnSOD persisted in the absence of the SirT1 gene and in the presence of the sirtuin inhibitor sirtinol. However, the deletion of the SirT1 gene generally reduced cellular stress resistance (data not shown), as might be expected based on known interactions of SirT1 with the stress response transcription factor HSF-1 (Westerheide et al., 2009). Disruption of HSF-1 impairs the expression of stress-inducible heat shock proteins in mice, and negatively affects their ability to withstand environmental stressors (reviewed in Pirkkala et al., 2001). Thus, the deletion of the SirT1 gene probably had many effects on cellular stress resistance independent of RES. Similarly, SirT1 independent effects of RES have been previously reported. For example, Mader et al. (2010) recently demonstrated that RES promotes apoptosis in human preadipocytes when SirT1 expression has been decreased using RNA interference (Mader et al., 2010). RES's effects on insulin sensitivity were also shown to be independent of SirT1 (Fullerton and Steinberg, 2010). Protein deacetylation by sirtuins has recently been shown to regulate enzymes involved in mitochondrial fatty-acid oxidation (Hirschey et al., 2010) and may therefore generally affect mitochondrial function. Given the newly identified regulatory role of protein acetylation (reviewed in Lu et al., 2009), it is likely that SirT1 gene deletion has substantial and wide-ranging effects on cell biology. This observation, and the difficulties in confirming the allosteric activation of sirtuins by RES, suggests that sirtuin activity may tangentially affect, but not direct, RES's biological activities.

In addition to RES's affect on MnSOD, an increase in the activity of a second antioxidant enzyme, glutathione peroxidase was also observed in cells treated with RES. Glutathione peroxidase is responsible for the reduction of hydrogen peroxide, and exists as multiple isoforms within the cell (see Margis et al., 2008 for review). Glutathione peroxidase 1 is the most abundant isoform, however, the phospholipid hydroperoxide glutathione peroxidase 4 plays an important role in stress resistance. Overexpression of glutathione peroxidase 4 is capable of protecting mouse fibroblasts from hydrogen peroxide induced cell death (Dabkowski et al., 2008). In contrast, fibroblasts that are heterozygous for glutathione peroxidase 4 display a higher sensitivity to hydrogen peroxide (Garry et al., 2008). Unfortunately, it was not possible to distinguish between glutathione peroxidase 1 and glutathione peroxidase 4 activity in the samples collected here. Further research into which glutathione peroxidase isoform is affected by RES is necessary.

Prior to RES's reported activation of sirtuins, it was identified as a phytoestrogen (Gehm et al., 1997) capable of binding estrogen receptors alpha and beta with Ki values in the low micromolar range (Bowers et al., 2000). The ability of RES and other phytoestrogens to affect estrogen signaling may explain how the compounds can have a positive effect on health, in spite of relatively low bioavailability (Wenzel and Somoza, 2005a). Interestingly MnSOD, and mitochondria more generally, are important downstream targets of estrogen signaling (reviewed in Klinge et al., 2008). Similar to what is observed with RES (Robb et al., 2008a), estrogen has been reported to increase apparent mitochondrial number in a variety of cell types (reviewed in Chen et al., 2009). Estrogen signaling has been demonstrated to increase the transcription of both MnSOD

and glutathione peroxidase in MCF7 cells (Borrás et al., 2003). Mitochondria isolated from females also express higher levels of MnSOD than males (Borrás et al., 2005), supporting the hypothesis that estrogen decreases mitochondrial ROS production and protects against oxidative stress. The effects of RES on cellular stress resistance and MnSOD could be inhibited by treatment with the estrogen inhibitor ICI 182780 in C2C12 cells, which are known to possess estrogen receptors (Milanesi et al., 2009), suggesting that estrogen signaling is essential to RES's effect. It has been hypothesized that the protective effects of phytoestrogens are mediated through ERbeta (Viña et al., 2008). While RES binds to both ERalpha and ERbeta with similar affinity, it appears to exert strong transcriptional effects via ERbeta (Bowers et al., 2000). The ERbeta agonist DPN, but not the ERalpha agonist PPT was capable of inducing an increase in MnSOD protein level. ERbeta is expressed in particularly high levels in brain tissue (Taylor and Al-Azzawi, 2000), and it is interesting that RES can increase MnSOD specifically in brain (Robb et al., 2008b; Chapter 6).

RES belongs to a family of phytoestrogens that are capable of binding to ERbeta, and may have the ability to induce similar effects (Viña et al., 2008). For example, many similarities between the biological activities of RES and genistein exist, including observations of increased MnSOD activities (Viña et al., 2008). It will be interesting to determine whether other phytoestrogens are capable of eliciting the same positive effects on health as RES.

Chapter 3: Resveratrol and Estrogen Receptor Beta Agonist DPN Affect Mitochondrial Morphology

Hypothesis: RES and the ERbeta agonist DPN reduce proliferative cell growth and confer cytoprotection, two cellular properties that are consistent with hyperfusion of the mitochondrial reticulum. It is hypothesized that both RES and DPN impinge upon the fusion state of the mitochondrial reticulum, and that this change requires an induction of MnSOD.

Objectives: The objectives of this project were to evaluate the effects of RES and ERbeta agonists on mitochondrial morphology.

Contributions:

I performed all experiments, statistical analysis and manuscript prepration.

3.1 Introduction

A number of positive health effects have been attributed to the phytoestrogen RES, including neuroprotection, cardioprotection, and anti-cancer activity (Baur and Sinclair, 2006; Robb and Stuart, 2010). Details of the molecular mechanisms responsible for RES's effects have come mostly from experiments involving rodents and *in vitro* work with cultured cells. RES's neuroprotective and cardioprotective activities in rodents can be modelled in a diverse collection of cell types *in vitro* as cytoprotection against exogenous toxins including paraquat, hydrogen peroxide and the DNA alkylating agent methyl methane sulfonate (MMS) (Robb and Stuart, 2011; Chapter 2). Consistent with its anti-cancer activity RES inhibits cell cycle progression and proliferative growth in cancerous, but also in normal cell lines (Bishayee et al., 2009).

Mitochondria are an important site of RES's actions, which include changes in ROS metabolism, bioenergetics and biogenesis. In mice a dietary supplement of RES included in a high fat diet significantly increases markers of mitochondrial abundance in skeletal muscle and liver tissue (Lagouge et al., 2006; Baur et al., 2006). A similar increase in mitochondrial abundance and specific mitochondrial proteins is observed in cultured endothelial cells, myoblasts, fibroblasts and neuroblastomas treated with RES (Baur et al., 2006; Csiszar et al., 2009; Robb and Stuart, 2011; Chapter 2). While many of RES's mitochondrial effects have been well characterized, one variable property that has not been investigated within the framework of RES's molecular mechanism is mitochondrial morphology.

Mitochondria exist in a dynamic equilibrium that fluctuates between fragmented, discrete organelles and a fused, continuous network. The structure of the mitochondrial

network is influenced by intracellular redox status and energy availability (reviewed in Youle and Van der Bliek, 2012). A highly fused reticulum can impede progression through the cell cycle, while a fragmented reticulum is observed in rapidly dividing cells including cancerous cell lines (Mitra et al., 2009; Rehman et al., 2012). The morphology of the mitochondrial reticulum also impacts upon cellular stress resistance by promoting electrical continuity and complementarity between mitochondrial components. Pharmacological inhibition of mitochondrial fission prevents cardiomyocyte death from ischemia reperfusion *in vitro* and can reduce the amount of damage accrued following coronary artery obstruction in mice (Ong et al., 2010). Interestingly, mitochondrial hyperfusion shares two key cellular characteristics with RES treated cells: slowed mitotic cell growth and enhanced cytoprotection.

The regulatory factors and signaling pathways that direct the ultrastructure of the mitochondrial network are just beginning to be elucidated. It was recently shown that the morphology of the mitochondrial reticulum is impacted by the intracellular redox state in animal cells (Shutt et al., 2012). The activities of enzymes responsible for mitochondrial fusion are altered by redox modification of critical cysteine residues in response to changes in intracellular concentrations of reduced glutathione, an important determinant of the cellular redox environment (Shutt et al., 2012). A more oxidized environment is conducive to an increase in mitochondrial fusion, while a more reduced intracellular environment stimulates fission (Shutt et al., 2012). The mechanisms responsible for this observation are currently unknon; however, the intracellular redox state is an important determinant of mitochondrial morphology.

In addition to stimulating changes in mitochondrial abundance, RES treatment also alters ROS metabolism. RES induces an upregulation of MnSOD protein levels and activity that is concomitant with cytoprotection and inhibition of mitotic growth in a variety of cell types (Robb et al., 2008; Robb et al., 2008b; Robb and Stuart, 2011; Ryan et al., 2010; Fukui et al., 2010; Kairisalo et al., 2010; Ungvari et al., 2009). Critically, when MnSOD induction is prevented these cellular effects are absent (Robb and Stuart, 2011; Fukui et al., 2010; Chapter 2). MnSOD localizes exclusively to the mitochondrial matrix where it catalyzes the dismutation of superoxide anions produced during aerobic respiration to hydrogen peroxide, which may then be converted to water via several mitochondrial and cytosolic pathways. The only enzymatic activity that has been ascribed to MnSOD is its role in ROS metabolism, and it is plausible that mitochondrial ROS metabolism via MnSOD activity is essential to RES's cytoprotective and growth inhibitory effects. The mechanism by which ROS metabolism influences cell proliferation and cytoprotection has not been conclusively identified, but may involve a redox mediated change in mitochondrial morphology.

Given the similarities between the effects of RES and hyperfusion in mammalian cells, it is hypothesized that RES modulates the morphology of the mitochondrial reticulum. To explore this idea the effects of RES on mitochondrial fusion were evaluated in mouse myoblasts (C2C12), primary mouse myoblasts, human lung fibroblasts (MRC5), and human prostate cancer cells (PC3). RES was identified as a phytoestrogen in 1997 (Gehm et al., 1997; Bowers et al., 2000), and in agreement with this property of RES, in the presence of ER antagonist ICI182780 RES fails to induce MnSOD, and in turn its cytoprotective and anti-proliferative effects are largely

eliminated. As ERbeta agonists replicate RES's cytoprotective and growth inhibitory effects, experiments to evaluate mitochondrial morphology were also performed in primary myoblast lines derived from ERbeta null mice.

3.2 Experimental Procedures

3.2.1 Materials:

Modified Eagle Medium with Earle's salts, l-glutamine and sodium bicarbonate, Dulbecco's Modified Eagle Medium with high glucose, l-glutamine and sodium bicarbonate and Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 were obtained from Sigma–Aldrich (St. Louis, MO). Penicillin/streptomycin, non-essential amino acids, and fetal bovine serum were obtained from Hyclone (Logan, UTtah). Puromycin was obtained from BioShop (Burlington, ON). RES was obtained from A.G. Scientific (San Diego, CA). Mdivi-1 and Diarylpropionitrile (DPN) were obtained from Tocris Bioscience (Ellisville, MO). Propylpyrazole triol (PPT) was obtained from Cayman Chemicals (Ann Arbor, MI). Bradford BioRad Protein Assay dye was purchased from BioRad laboratories (Hercules,CA). Prestained broad range protein marker was purchased from BioLabs (New England, MA). Pierce Memcode Reversible Protein Stain Kit^{TM} was obtained from Thermo Fisher Scientific (Mississauga, Canada). Drp1 antibody was purchased from BDBiosceinces (Mississauga, ON). Phospho-Drp1 Ser637antibody was purchased from Cell Signaling (Danvers, MA). Infrared dye-conjugated secondary antibodies to rabbit and mouse were purchased from Rockland Immunochemicals (Gilbertsville, PA). Porcine trypsin and soybean trypsin inhibitor were purchased from BioShop (Burlington, ON). Renilla luciferase kit was purchased from Promega (Madison, WI). CellLight mtGFP was purchased from Life Technologies (Burlington, ON). All other chemicals and purified enzymes were obtained either from

Sigma–Aldrich (St. Louis, MO), BioShop (Burlington, Canada) or Fisher Scientific (Mississauga, Canada) unless otherwise stated.

3.2.2 Cell Lines and Culture Conditions

MRC5 (Coriell), C2C12 (Sigma) and PC3 (ATCC) cell lines were cultured in accordance with the distributor's protocol. HeLa cell lines expressing N or C Mito venus-zipper-luciferase (mitoVZL) were a gift from Dr. Heidi McBride at McGill University. They were generated and cultured as described in Shauss et al., 2010. ERbeta null and control myoblast cell lines were generated from 5 individuals each of wildtype and ERbeta-/- mice in a C57/BL6 background (Taconic Farms, Hudson, NY, USA) (Krege et al., 1998). Myoblasts were isolated as in Robb et al., (2012). Briefly, to isolate primary myoblasts hind limb skeletal muscle was excised, finely minced and suspended in pronase solution (2mg/mL) at 37ºC for 1h. The suspension was titurated by repeated pipetting and then filtered through sterile cheesecloth and centrifuged at 240g for 5 minutes. The resulting supernatant was then centrifuged at 500g for 5 minutes. The pellet was resuspended in growth media (Ham's F10 nutrient mixture, 20% FBS, nonessential amino acids, penicillin, streptomycin, gentamicin, amphotericin, basic human growth factor), plated on to collagen coated tissue culture dishes and incubated at 37ºC, 3%O_2 and 5%CO_2. An equal volume of media was added 48h after plating, and changed 96h after plating. Myoblast outgrowth was evident at 96h. Myoblasts were subcultured at a ratio of 1:3 when they reached 70% confluence. All cell lines were cultured under conditions of 37 °C, humidified to 5% CO_2, 18% O_2 atmosphere, with the exception of primary myoblasts that were cultured under 3% O_2. Cell density was determined by counting. Treatments were added directly to culture media and refreshed daily.

3.2.3 Cell Microscopy

Mitochondria were imaged following transfection with CellLight mtGFP, as per the manufacturer's instructions. Cells were fixed 18h after transfection. Briefly, the fixation protocol consisted of incubation with a 4% (w/v) paraformaldehyde solution at 37°C for 10 minutes followed by a 20 minute incubation with 1M ammounium chloride at room temperature and repeated rinsing in phosphate buffered saline. Cells were imaged immediately, or stored at 4°C in sterile PBS. Images were acquired using a Leica DM IRE2 microscope or an Olympus FluView 300 confocal microscope, 63× NA1.4 objective lens, equiped with a 488nm laser to visualize the GFP. Mitochondrial morphology was characterized as fragmented or tubular. The percentage of cells with the indicated morphology was calculated as the percentage of the total cells imaged (>25 per experiment) (as outlined in Rambold et al., 2011). Images were analyzed using ImageJ software and Adobe Photoshop CS2.

3.2.4 Preparation of Whole Cell Lysates

Cells were lysed using the method outlined in Chapter 2. The protein concentration of the resulting supernatant was determined as outlined in Chapter 2. Whole cell lysates were stored at -80˚C.

3.2.5 Mitochondrial and cytosol purification

Cells (including N and C mitoVZL expressing HeLa) were harvested by trypsinization. Whole cells were washed two times in cold mitochondrial isolation buffer (MIB) containing 220mM Mannitol, 68mM sucrose, 80mM KCl, 0.5mM EGTA, 2mM $Mg(CH3COO)_2$, 2mg/mL fatty acid free BSA and 10mM HEPES at pH 7.4. Cells were

resuspended in 1.5x volumes of cold MIB, and homogenized using a chilled glass dounce homogenizer. The homogenate was centrifuged at 3000g for 10 minutes at 4°C and the supernatant was isolated and maintained at 4°C (post-nuclear supernatant PNS). The pellet was washed in 1x volume of cold MIB and centrifuged at 3000g for 10 minutes at 4°C, and the resulting PNS was isolated. Mitochondria were isolated from the PNS by centrifugation at 9000g for 15 minutes at 4°C. The supernant was collected as the cytosolic fraction and was further cleared by centrifugation at 20000g for 1h at 4°C. The cytosols were aliquoted and snap frozen in liquid nitrogen. The pellet containing mitochondria was washed once in MIB and centrifuged at 9000g for 15 minutes at 4°C. The isolated mitochondria were then resuspended in MIB without BSA and with 10% (v/v) glycerol, were aliquoted and snap frozen in liquid nitrogen.

3.2.6 Western Blots

Western blotting was performed as outlined in Chapter 2. 20µg of protein was used unless stated otherwise. AntiDrp1 was used at a 1:500 dilution. AntiDrp1-P was used at a 1:250 dilution.

3.2.7 Cell free mitochondrial fusion assay

The *in vitro* assay of mitochondrial fusion was performed as described in Schauss et al., 2010. Briefly, 50µg of C and N mitoVZL mitochondria were incubated in a reaction containing 10mM HEPES pH7.4, 110mM mannitol, 68mM sucrose, 80mM KCl, 0.5mM EGTA, 2mM $Mg(CH_3COO)_2$, 0.5mM GTP, 2mM K_2HPO_4, 1mM ATP, 0.08mM ADP, 5mM Na succinate, 1mM DTT and 5mg/mL cytosol. The mitochondria were concentrated by centrifugation, and were incubated on ice for 30 minutes. The

mitochondria were then resuspended and incubated in a 37°C water bath for 30 minutes. Signal arising from ruptured mitochondria was quenched by incubation with 25µg trypsin for 15 minutes on ice, after which trypsin activity was inhibited by incubation with 500µg soy bean trypsin inhibitor for 15 minutes on ice. Mitochondria were concentrated by centrifugation at 9000g for 1 minute, and solublized by 1h incubation with 50µL of lysis buffer (Promega) on ice. Luciferase activity was detected using a commercially available renilla luciferase kit (Promega) and fluorescence was detected using a Varian Cary Eclipse fluorescence spectrometer.

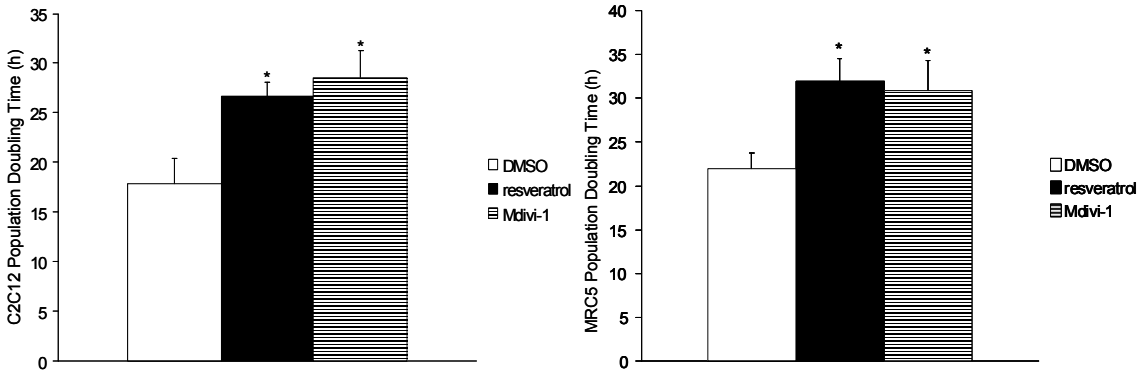
3.2.8 Statistical Analysis:

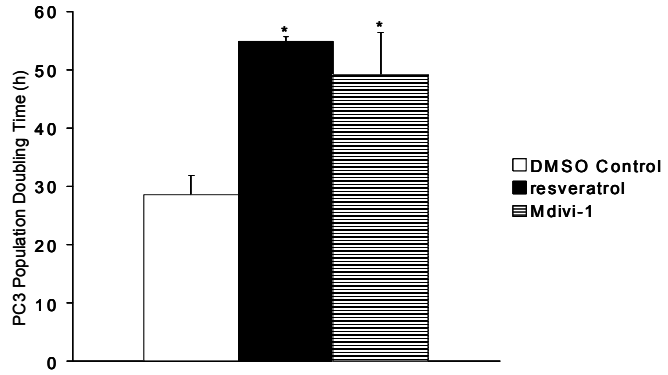
Data were analyzed by ANOVA using Systat v.12. Post-hoc comparisons between means were performed using Tukey's test. Data comparing two experimental groups were analyzed using the student's t-test. All data are presented as means ± standard error of the mean (SEM). A p-value of < 0.05 was considered significant.

3.3 Results

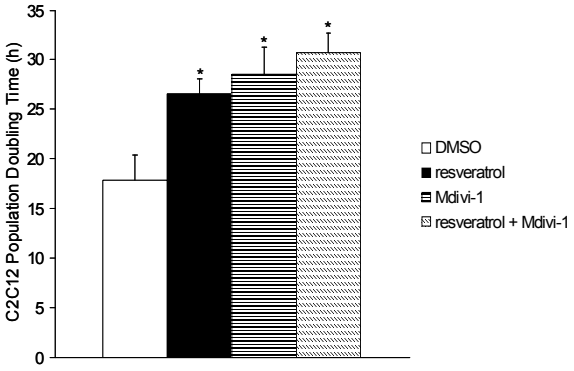
As a preliminary experiment to investigate whether a stimulation of mitochondrial fusion could mimic the antiproliferative effect of RES, myoblast (C2C12), fibroblast (MRC5) and prostate cancer (PC3) cells were treated with 10μM mdivi-1, a direct inhibitor of the fission enzyme Drp1 (Cassidy-Stone et al., 2008) for 72h. Mdivi-1 significantly slowed the proliferative growth rates of all three cell lines by an amount similar to that achieved with 20μM RES (Fig. 3.1A). To explore the possibility that mdivi-1 and RES impede cell growth through different cellular mechanisms, the effect of simultaneous addition of mdivi-1 and RES on proliferative cell growth was tested in C2C12 myoblasts. An additive effect on growth rate was not observed (Fig. 3.1B), suggesting that perhaps both compounds were working via a shared pathway involving mitochondrial fusion. In C2C12 cells, mdivi-1 was protective against hydrogen peroxide toxicity, similar to what is achieved with RES treatment (Fig. 3.1C). MnSOD induction was quantified in C2C12 cells treated with RES and with mdivi-1 and, while the typical induction of MnSOD by RES was observed, mdivi-1 had no effect (Fig 3.1D).

3.1 A

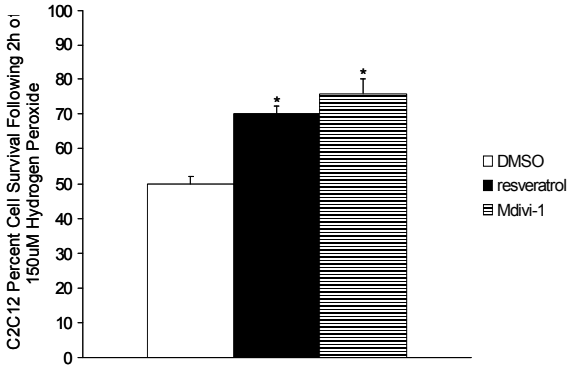




3.1 B



3.1 C



3.1 D

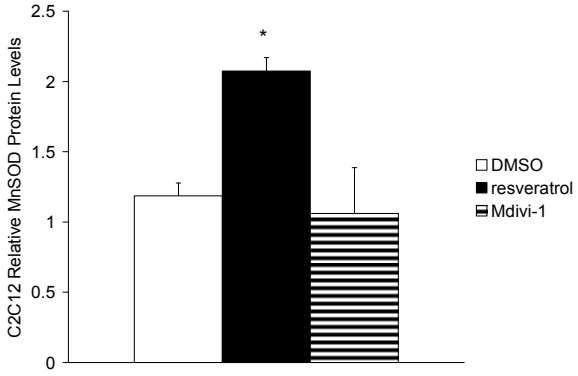
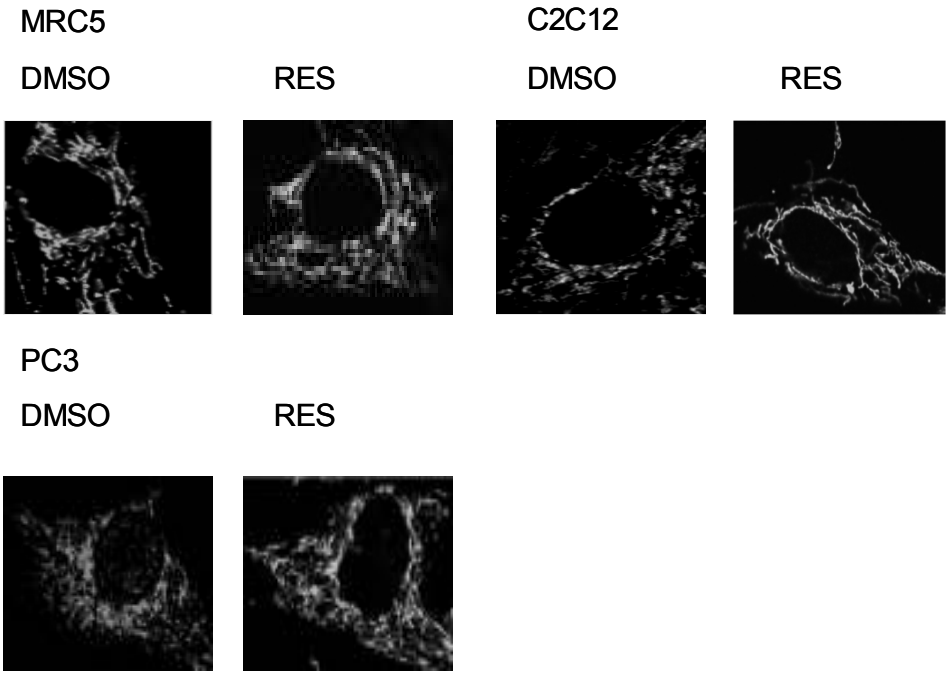


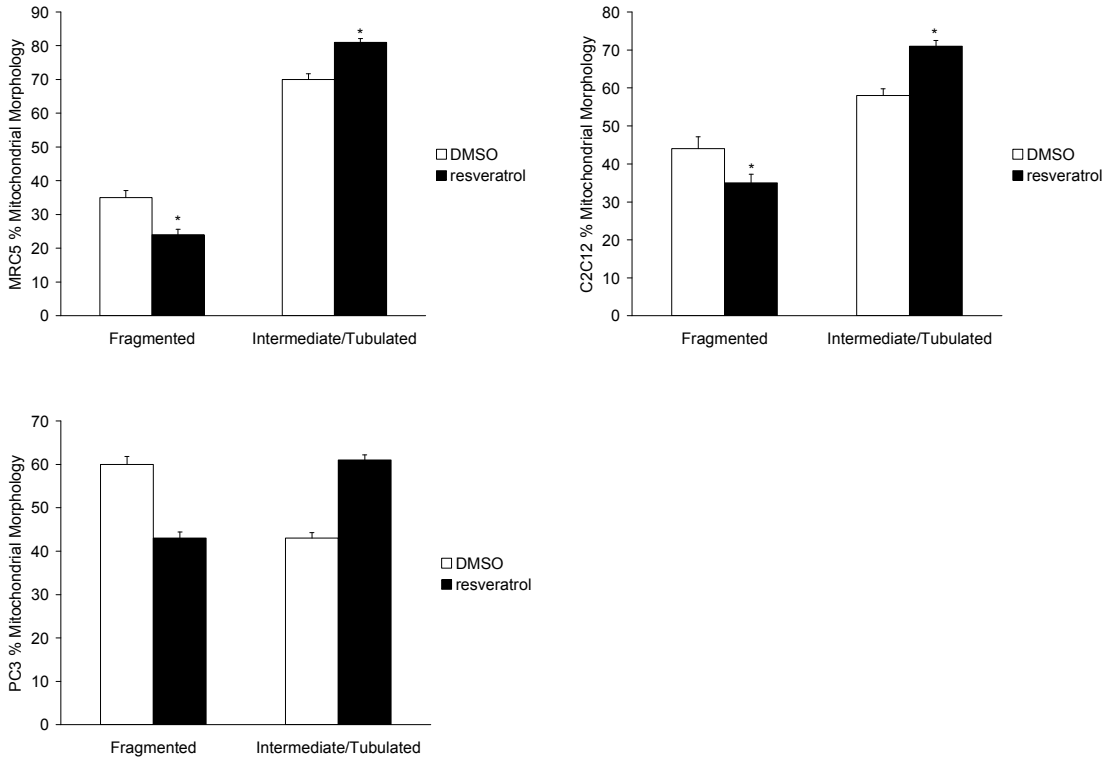
Figure 3.1. Inhibition of mitochondrial fission mimics the effects of RES on population doubling times and stress resistance, but not MnSOD protein levels. A. Effect of 48h treatment with 20μM RES or 10μM Mdivi-1 on population doubling time in MRC5, C2C12 and PC3 cell lines. B. Effect of 48h treatment with 20μM RES and 10μM Mdivi-1 on population doubling time in C2C12 cell line. C. Percentage of living cells following 2h exposure to 150μM H2O2 in C2C12 cells pre-treated with DMSO (vehicle control), 20μM RES or 10μM Mdivi-1 for 48h. D. Relative MnSOD protein levels in C2C12 cells treated with 20μM RES or 10μM Mdivi-1. n=3. Error bars represent SEM. *=$p<0.05$ compared to control.

To visualize the effects of RES on mitochondrial morphology MRC5, C2C12, and PC3 cells were treated with RES for 48h, transfected with a mitochondrial-targeted green fluorescent protein (mtGFP) and imaged using confocal microscopy (Fig. 3.2A). Images were evaluated and quantified as tubular (>4.5μm) or fragmented (<4.5μm) (Fig. 3.2B). RES treatment increased the proportion of 'tubulated' and decreased the proportion of 'fragmented' mitochondria. To corroborate these results, a biocomplementation assay of mitochondrial fusion (Shauss et al., 2010), was performed with cytosol extracts prepared from MRC5 and C2C12 cells treated with RES or vehicle control (DMSO). In this 'cell-free' *in vitro* fusion assay, luciferase luminescence results when two populations of mitochondria share contents to produce a single functional luciferase protein that reports fusion. This method produced similar results, showing a stimulation of fusion in the cytosols from RES-treated cells (Fig. 3.2C).

3.2 A



3.2 B



3.2 C

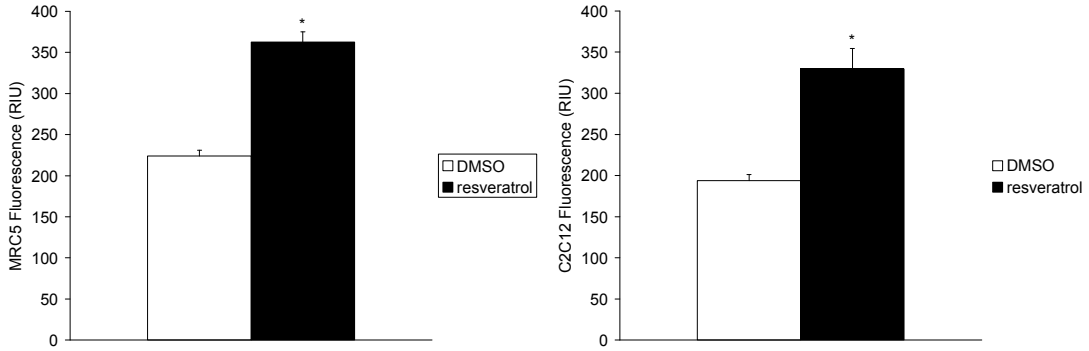


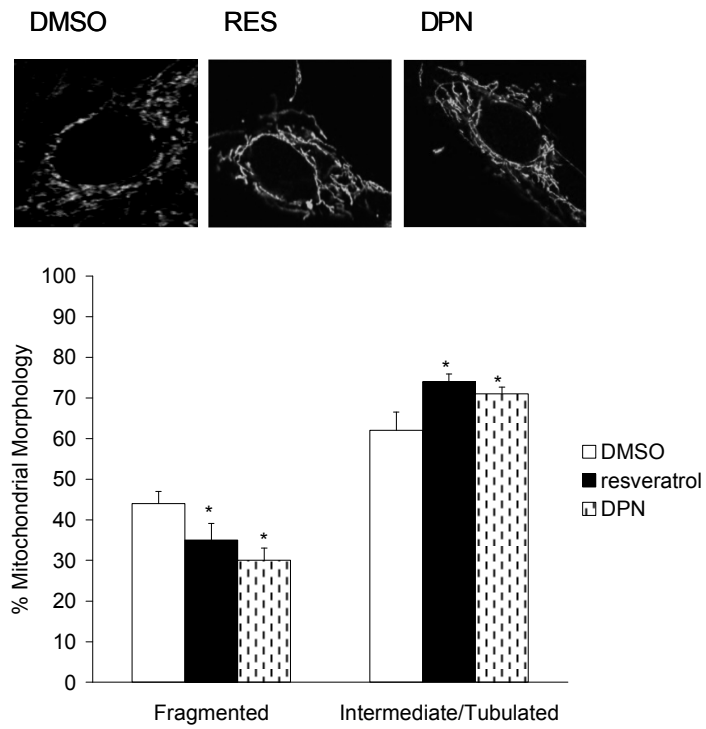
Figure 3.2. Resveratrol stimulates mitochondrial fusion.
A. Mitochondrial morphology in MRC5, C2C12 and PC3 cells treated with DMSO (vehicle control) or 20μM RES for 48h visualized using a mitochondrial targeted GFP. B. Quantification of mitochondrial morphology in MRC5, C2C12 and PC3 cells treated with DMSO (vehicle control) or 20μM RES for 48h. >50 cells per treatment group. C. Stimulation of fusion measured by a cell free fusion assay in MRC5 and C2C12 cells treated with DMSO (vehicle control) or 20μM RES for 48h. n=3. Error bars represent SEM. *=$p<0.05$ when compared to control.

Many of RES's cellular activities are consistent with its actions as a phytoestrogen. The ERbeta agonist DPN, but not the ERalpha agonist PPT, can elicit an induction of MnSOD expression and the associated changes in stress resistance and proliferative cell growth in cultured myoblasts (Robb and Stuart, 2011; Chapter 2). To determine if the ERbeta agonist DPN shared RES's ability to impinge upon the structure of the mitochondrial network, mitochondria were visualized in DPN treated myoblasts. Consistent with the hypothesis that ERbeta is a central component of RES's molecular mechanism, DPN treatment stimulated fusion nearly as strongly as RES (Fig. 3.3A). Cytosols of C2C12 myoblasts treated with DPN stimulated fusion, while cytosols treated with the ERalpha agonist PPT had no effect in the cell free mitochondrial fusion assay (Fig. 3.3B).

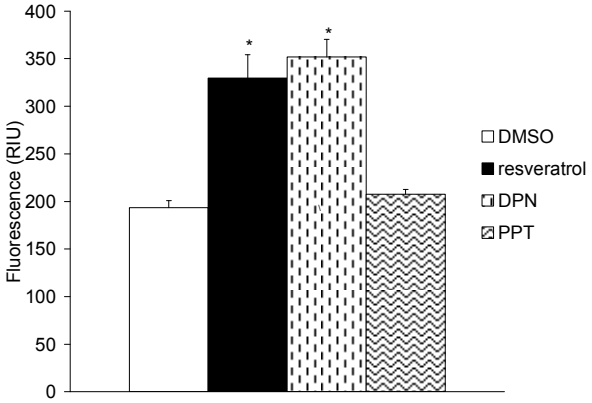
Pedram and colleagues (2006) showed that estrogen could elicit rapid effects on redox metabolism in isolated mitochondria. To test if the stimulation of fusion by DPN and RES observed in the cell free fusion assay was due to a direct interaction between these compounds and the isolated mitochondria, RES and DPN were added directly to the *in vitro* fusion assay. RES or DPN addition directly to the cell free assay did not stimulate mitochondrial fusion (Fig. 3.3C).

To confirm the role of ERbeta, the effects of both RES and DPN on mitochondrial fusion were determined in ERbeta null myoblasts. Neither compound stimulated fusion in the absence of ERbeta (Fig. 3.3D), indicating that this receptor plays an important role in the observed effect on mitochondrial morphology.

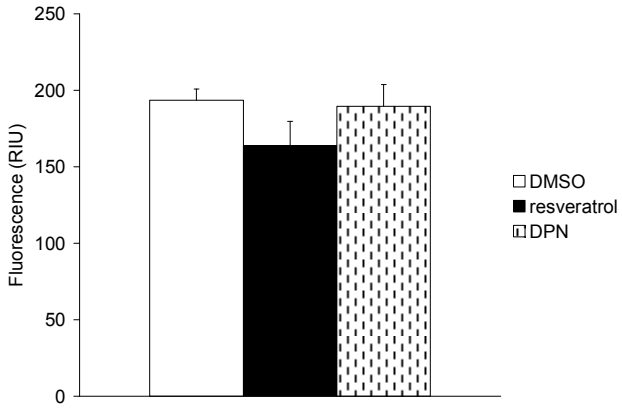
3.3 A


C2C12
DMSO
RES
DPN
% Mitochondrial Morphology
100
90
80
70
60
50
40
30
20
10
0
Fragmented
Intermediate/Tubulated
DMSO
resveratrol
DPN

3.3 B


Fluorescence (RIU)
400
350
300
250
200
150
100
50
0
DMSO
resveratrol
DPN
PPT

3.3 C



3.3 D

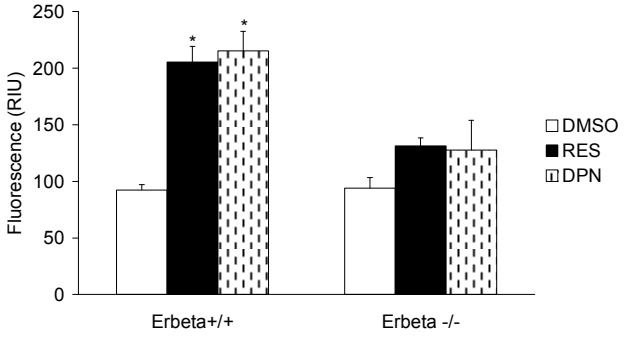
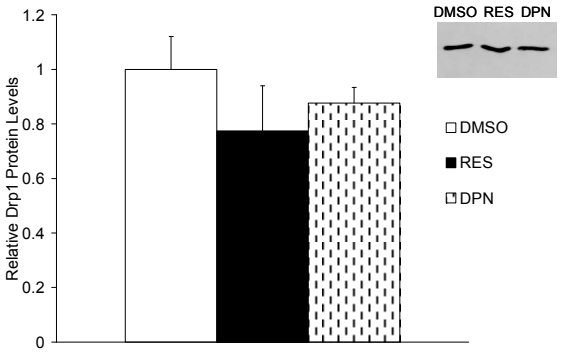


Figure 3.3. ERbeta is necessary for resveratrol to stimulate mitochondrial fusion. A. Mitochondrial morphology in C2C12s treated with DMSO, 20μM RES or 10μM DPN for 48h. >25 cells imaged per treatment. B. Effect of cytosols from C2C12 cells treated with DMSO, 20μM RES, 10μM DPN or 10μM PPT for 48h on fusion evaluated using the cell free fusion assay C. Impact of 1μM RES or DPN fusion measured by the cell free fusion assay. D. Effect of cytosols from ERbeta+/+ and ERbeta-/- myoblasts treated with DMSO, 20μM RES or 10μM DPN for 48h on fusion evaluated using the cell free fusion assay. n=3. Error bars represent SEM. *=$p<0.05$ compared to control.

The fusion state of the mitochondrial reticulum represents the balance between fusion and fission events and may therefore be influenced by regulation of proteins that catalyze either or both processes. While the exact compliment of proteins required for fission and fusion has not been definitively determined, the dynamin-related protein Drp1

appears to be a particularly important regulator of fission. Total Drp1 protein levels in myoblasts treated with DMSO, RES or DPN were quantified by Western blot, but did not differ signficantly between groups (Fig. 3.4A). One mechanism of Drp1 regulation is an inhibition of its activity by the specific phosphorylation of serine 637. Therefore, the levels of phospho-Drp1(S637) were investigated by Western blot. While the signal intensity of the phospho-Drp1(S637) was low, based on qualitative observation the RES and DPN treated groups had higher levels of phospho-Drp1(S637) than the vehicle control (Fig. 3.4B).

3.4 A



3.4 B

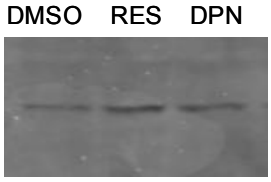


Figure 3.4. RES and DPN do not affect the total protein level of Drp1.
A. Protein level of Drp1 in whole cell lysates of C2C12 treated with DMSO, 20μM RES or 10μM DPN for 48h. B. Representative western blot for detection of Phospho Drp1 in C2C12 treated with DMSO, 20μM RES or 10μM DPN for 48h. n=3. Error bars represent SEM. *=$p<0.05$ when compared to control.

3.4 Discussion

Fusion of the mitochondrial reticulum is essential to maintaining mitochondrial function (reviewed in Detmer and Chan, 2007). In cells lacking the fusion enzymes Mfn1 and Mfn2 there is a progressive loss of mtDNA, and in turn a reduction in oxidative metabolism and mitochondrial membrane potential (Chen et al., 2005). *In vivo* mitochondrial fusion appears to be particularly important to the health of highly metabolic tissues, including nervous tissue, cardiac and skeletal muscle. Mice with a mutation in Mfn2 display symptoms of severe neurodegeneration (Chen et al., 2007), and in humans a similar mutation in Mfn2 underlies the neurodegenerative phenotype of Charcot-Marie-Tooth type 2A disease (Zuchner et al., 2004). In skeletal muscle, a conditional knockout of Mfn1 and Mfn2 leads to severe respiratory defects and high levels of muscle atrophy in mice (Chen et al., 2010). Mitochondrial fusion is also implicated in the maintenance of energy homeostasis and insulin signaling. Reduced protein levels of Mfn2 are observed in patients with type two diabetes (Hernández-Alvarez et al. 2010), and a liver specific deletion of Mfn2 leads to glucose intolerance and aberrant insulin signaling in mice (Sebastian et al., 2012).

An increase in mitochondrial fusion is generally cytoprotective, and is associated with neuroprotection and resistance against cardiac injury arising from ischemia reperfusion. For example, in cultured cerebellar granule neurons an increase in mitochondrial fusion mediated by induction of the fusion enzyme Opa1 is protective against glutamate toxicity (Jahani-Asl et al., 2011). Increased fusion stimulated by a reduction in Drp1 activity in a cardiac cell line (HL-1) and in cardiomyocytes prevents cell death in a model of ischemia reperfusion. This same protective effect of reduced

fission is observed *in vivo*, where treatment with the Drp-1 inhibitor mdivi-1 protects against cardiac cell loss following coronary artery occlusion and reperfusion (Ong et al., 2011). Thus, increased mitochondrial fusion can protect against stress-induced cell death.

A further beneficial effect of mitochondrial fusion is its ability to slow the growth of cancerous cells. A hyperfused mitochondria reticulum is observed in the G_1-S transition in the cell cycle, and maintenance of this tubular state can prevent mitosis (Mitra et al., 2009). In contrast, a highly fragmented mitochondrial network, similar to what was observed in the prostate cancer cell line (PC3) used here, is observed in metastic breast cancer cell lines (Zhao et al., 2012). A reduction in fission achieved using an inhibitor of Drp1 reduces the growth of cancerous breast and lung cell lines (Zhao et al., 2012; Rehman et al., 2012). In this study, mdivi-1 significantly slowed the proliferative cell growth of fibroblasts, myoblasts and prostate cancer cells, presumably by a eliciting a change in the fission and fusion state of the mitochondrial reticulum.

Overall, a reduction in mitochondrial fusion is associated with increased cell loss and tissue dysfunction, while an increase in fusion is associated with cytoprotection and an inhibition of proliferative cell growth. It is therefore interesting that RES and DPN, two compounds that are neuroprotective (*i.e.* Simao et al., 2011; Aguirre et al., 2010), cardioprotective (*i.e.* Bradamante et al., 2003; Hsieh et al., 2006), have positive effects on metabolic health (*i.e.* Lagouge et al., 2006; Yepuru et al., 2010) and anti-cancer activities (*i.e.* Jang et al., 1997; Motylewska et al., 2008) stimulate mitochondrial fusion in fibroblasts, myoblasts and in prostate cancer cells. While the effects of RES and estrogens on other mitochondrial measures including ROS metabolism and biogenesis

have been well established, this study provides the first evidence in support of the hypothesis that these compounds also impact the dynamic between mitochondrial fission and fusion.

Although the exact mechanism that lead to the enhanced fusion was not established here, the effects of RES and mdivi-1 on population doubling time were not additive, which is consistent with a model in which these two compounds work through a shared molecular mechanism. Mdivi-1 interacts directly with Drp1 to inhibit its catalytic activity (Cassidy-Stone et al., 2008). While it is unlikely that RES is interacting directly with Drp1, the activity of this enzyme is regulated by a number of posttranslational modifications including phosphorylation, sumoylation, ubiquitination and nitrosylation that may be downstream targets of pathways affected by RES (reviewed in Chan, 2012). Phosphorylation of Drp1 at serine 637 inhibits its fission promoting activity (Chang and Blackstone, 2010), and the increased levels of phosporylated Drp1 at Serine 637 observed in RES and DPN in myoblasts is consistent a more fused mitochondrial reticulum.

The role of the mitofusion proteins in the increased fusion observed with RES and DPN treatment was not explored in this thesis. Mfn2 is a downstream target of PGC-1alpha (Cartoni et al., 2005), which is a known target of RES (Lagouge et al., 2006). mRNA levels of mfn2 are increased by estradiol treatment in the MCF-7 breast cancer cell line, but it is unknown how this increase relates to protein levels (Sastre-Serra et al., 2012). Both a reduction in the activity of fission enzymes, and an increase in the activity of fusion enzymes can account for the observations of hyperfusion made here with RES and DPN. Evaluating the effect of these compounds on mfn2 levels will be an important goal of future research.

The ability of RES and DPN to promote fusion was dependent upon the presence of ERbeta. The presence of ERbeta in mitochondria has been reported by several research groups (Yang et al., 2004; Razandi et al., 2012), however the biological importance of this observation remains unclear. The addition of DPN directly to the cell free fusion assay did not promote fusion, which suggests that this molecule does not stimulate fusion via direct interactions with the mitochondrial ERbeta.

In conclusion, data from this study demonstrates that mitochondrial fusion is stimulated by RES and DPN in fibroblasts, myoblasts and prostate cancer cells. Given the role of mitochondrial fusion in cellular stress resistance and proliferative cell growth, this is likely to be an important effect of these compounds.

Chapter 4: Resveratrol's Effects on MnSOD, Proliferative Cell Growth and Stress Resistance are Shared by Structurally Similar Phytoalexins Found in *Vitis Vinifera*.

Hypothesis: RES's effects on cell proliferation, stress resistance and MnSOD will be shared by four structurally similar compounds: pterostilbene, piceid, resveratrol-4'-sulfate and resveratrol-3-glucuronide. ERbeta is essential to the mechanism of both RES and these three related compounds.

Objectives: The objectives of this project were to: **1)** Determine if four compounds with similar structures to RES increase MnSOD levels, reduce rates of cell proliferation and increase stress resistance, and **2)** Evaluate the importance of ERbeta in the effects of RES and related compounds using myoblast cell lines generated from an ERbeta null mouse.

Contributions:

I performed all experiments, statistical analysis and manuscript preparation.

4.1 Introduction

Phytoalexins are secondary metabolites produced by plants in response to environmental stress. In addition to their role in plant physiology, many of these compounds have biological activity in mammalian cells. RES is a phytoalexin that is found in the skin and leaves of wine grapes, and in other edible plants including peanuts and mulberries (reviewed in Soleas et al., 1997). RES's main dietary source is red wine, and it has been proposed that the cellular effects elicted by this molecule underlie many of the health benefits associated with red wine consumption (Siemann and Creasy, 1992). As an isolated compound, RES has been reported to have protective effects on metabolism, cardiovascular health, neurodegeneration and cancer (reviewed in Timmers et al., 2012). However, this purported therapeutic potential is limited by RES's low bioavailability, rapid metabolism and relatively low abundance in the human diet.

RES does elicit physiological effects *in vivo*, but the mechanism behind these effects is not well understood. In humans plasma levels of unmodified RES vary from undetectable to the low micromolar range as a consequence of extensive modification in the intestinal tract following its dietary intake (Wenzel and Somoza, 2005; Vitaglione et al., 2005). In contrast, concentrations of RES metabolites nearly ten fold higher than RES alone can be attained in plasma and tissues (Wenzel et al., 2005). Given that the levels of unmodified RES *in vivo* are quite low, there is uncertainty as to whether or not RES is the only active form of this compound. The *in vitro* experiments necessary to explore the hypothesis that RES's most prominent metabolites share its cellular effects have not been conducted.

In wine grapes (V*itis vinifera)* RES serves as a building block for a variety of compounds that are synthesized from its oligomerization, methylation or glycosylation. Two RES analogues identified in wine grapes are pterostilbene and piceid, which result from the methyoxylation and glycosylation, respectively, of RES. Interestingly, these compounds appear to share some of RES's biological activities in mammalian cells. Pterostilbene, a methylated compound with a high degree of structural similarity to RES, is effective at inhibiting the growth of cancerous cell lines *in vitro*. The anticancer properties of pterostilbene have been observed in cultured cancerous breast (MCF7; MDA-MB-231), colon (HCT116, HT29, Caco-2) and prostate cell lines (PC3, LNCaP) (Moon et al., 2012; Mannal et al., 2010; Nutakul et al., 2011; Lin et al., 2009). *In vivo*, dietary pterostilbene imparts protection against cognitive decline in a mouse model of accelerated aging used to approximate the conditions of Alzheimer's disease, and may therefore share RES's well established neuroprotective properties (Chang et al., 2012). Piceid, a second RES derivative abundant in red wines, also appears to be neuroprotective. In rats dietary piceid reduces oxidative stress and learning deficits following an ischemic event (Li et al., 2012). Thus, there is accumulating evidence that pterostilbene and piceid might share some of the key biological activities of RES. The possibility of mutual target enzymes and a shared molecular mechanism for these molecules has not been explored.

While a number of cellular pathways and molecular mechanisms have been put forth to account for RES's cellular effects, the antioxidant enzyme manganese superoxide dismutase (MnSOD) has been identified as playing a critical role in two important cellular effects of RES: reducing rates of proliferative growth and confering

cytoprotection in cultured fibroblast, myoblast and neuroblastoma cell lines (Robb et al., 2008a; Robb and Stuart, 2011; Chapter 2). Following the initial demonstration of increased MnSOD levels in response to RES, this observation has been repeated in skeletal muscle, cardiovascular cells, and in cultured neuronal cells (Jackson et al., 2010; Ungvari et al., 2009; Fukui et al., 2010; Sivritas et al., 2011). MnSOD is a mitochondrial protein that catalyzes the dismutation of the superoxide anion to hydrogen peroxide, and transgenic overexpression of MnSOD has been shown to influence cell proliferation and stress resistance (Ough et al., 2004; St Clair et al., 1991). Indeed, MnSOD is a powerful proximal regulator of the cell cycle (Sarsour et al., 2012). Given the role of MnSOD in mediating several key cellular effects of RES, it is plausible that structurally related molecules like pterostilbene and piceid are also working by this same mechanism.

Like RES, pharmacological agonists of ERbeta, such as diarylpropionitrile (DPN), are able to induce the expression and activity of MnSOD, inhibit cell replicative growth, and enhance cellular stress resistance. Regulation of MnSOD expression can be achieved by intracellular signaling pathways mediated by ERs (Sivritas et al., 2011), and RES is known to be a phytoestrogen that acts as an agonist for both ERalpha and ERbeta (Gehm et al., 1997; Bowers et al., 2000). It is hypothesized that increasing MnSOD protein levels is a shared mechanism between RES and other polyphenols found in red wine, mediated by ERbeta, that renders cells slow growing and stress resistant.

In this study the C2C12 myoblast cell line, which is well characterized and known to express ERs (Milanesi et al., 2008), mouse primary myoblasts, and the ERbeta expressing PC3 prostate cancer cell line were used to explore the cellular effects of RES derivatives and wine polyphenols. Two of the most prevalent human metabolites of RES:

trans-resveratrol-4'-sulfate (RES-S) and resveratrol-3'-O-β-D-glucuronide (RES-G), and two RES analogues that have greater bioavailability than RES and are abundant in red wine: pterostilbene and piceid were investigated for their effects on proliferative growth rate, cellular stress resistance and intracellular enzymes that can impart these qualities in cultured myoblasts. The activities of a these compounds were also explored in ERbeta null myoblasts to determine the role of this receptor in their molecular mechanism.

4.2 Experimental Procedures:

4.2.1 Materials

trans-RES was obtained from A.G. Scientific (San Diego, CA). trans-Piceid was purchased from Sigma (St. Louis, MO). trans-Resveratrol-4'-Sulfate sodium salt and trans-Resveratrol-3'-O-β-D-Glucuronide were purchased from Toronto Research Chemicals (Toronto, Canada). Pterostilbene was obtained from Cayman Chemical (Ann Arbor, MI). ICI182780, rolipram, apigenin, DPN and PPT were purchased from Tocris Biosciences (Ellisville, MO). All polyphenols purchased were of greater than 95% purity. Minimum Essential Medium, Dulbecco's Modified Eagle Medium with high glucose, Dulbecco's Modified Eagle Medium with high glucose without phenol red, Ham's F10 nutrient mixture, penicillin/streptomycin, non-essential amino acids, trypsin, fetal bovine serum, and charcoal stripped fetal bovine serum were obtained from Hyclone (Logan, UT). RPMI-1640 Medium without phenol red, gentamicin, amphotericin B, collagen, pronase and collagenase were obtained from Sigma–Aldrich (St. Louis, MO). BioRad protein dye was purchased from BioRad laboratories (Hercules, CA). Prestained broad range protein marker was purchased from Frogga Bioscience (Toronto, Canada). Pierce Memcode Reversible Protein Stain Kit^{TM} was obtained from Thermo Fisher Scientific (Mississauga, Canada). MnSOD antibody was purchased from Enzo Life Sciences (Brockville, Canada). CuZnSOD antibody and antibody to mouse hsp70 was purchased from Stressgen (Victoria, Canada). Hsp 60 antibody was purchased from Abcam (Cambridge, MA). mtTFAm antibody was purchased from Santa Cruz (Santa Cruz, CA). Infrared dye-conjugated secondary antibody to rabbit was purchased from Rockland Immunochemicals (Gilbertsville, PA). siRNA to MnSOD, Glyceraldehyde 3-

phosphate dehydrogenase (GAPDH), a scrambled control sequence, and NeoFx Transfection Agent™ were purchased from Ambion (Austin, TX). Cytotoxicity Detection Kit™ was purchased from Roche Applied Science (Laval, Canada). ERbeta null and control mice were purchased from Taconic Farms (Germantown, NY). SirT1 null and wild-type control mouse embryonic fibroblasts were kindly provided by Dr. Michael McBurney of the University of Ottawa (Ottawa, Canada). They were isolated as described in McBurney et al., (2003). The C2C12, MRC5 and PC3 cell lines were purchased from the American Type Culture Collection (Manassas, VA). All other chemicals and purified enzymes were obtained either from Sigma–Aldrich (St. Louis, MO), BioShop (Burlington, Canada) or Fisher Scientific (Mississauga, Canada) unless otherwise stated.

4.2.2 Cell Culture

The C2C12 mouse myoblast , MRC5 human lung fibroblast and PC3 prostate cancer cell lines were cultured at 37°C, 5% CO_2, 18% O_2 in accordance with the distributor's protocol and subcultured as required. SirT1 null and control mouse embryonic fibroblasts were cultured at 37°C, 5% CO_2, 3% O_2. ERbeta$^{-/-}$ and wildtype myoblasts were isolated as described in Robb et al., 2012 and Chapter 3. Primary myoblasts were cultured at 37°C, 5% CO_2, 3% O_2. PC3 cells were rendered rho^0 according to the protocol of (Joshi et al., 2009). Briefly, PC3 cells were cultured for eight weeks in the presence of 100ug/mL pyruvate, 50ug/mL uridine and 200ng/mL ethidium bromide. After eight weeks ethidium bromide was no longer added to the culture medium. Rho^0 status was confirmed by measures of cellular oxygen consumption and western blot for mtTFAM.

4.2.3 Resveratrol Analogue and Estrogen Antagonist Treatments

Each polyphenol was tested at 1, 10, 20 or 25, and 50μM to evaluate possible toxicity and to determine the concentration required for maximal effects on population doubling time. All polyphenols were dissolved in DMSO, with the exception of pterostilbene at 20μM, which was dissolved in 95% ethanol. In all cases media was refreshed daily with freshly prepared polyphenols. All treatments were of 48h durations. Comparisons were made to the appropriate vehicle control for each compound and the data presented for each compound correspond to the concentration of that compound that yielded maximal effects on population doubling time. Where the estrogen antagonist ICI182780 was used, it was added directly to the culture media at a final concentration of 10μM 24h prior to polyphenol treatment. This concentration was maintained throughout the 48h incubation period and was also refreshed daily.

4.2.4 siRNA Treatment

MnSOD knockdown experiments were conducted as described in Chapter 2.

4.2.5 Stress Resistance and Death Experiments

Stress resistance in myoblasts was evaluated as described in Chapter 2.

4.2.6 Lactate Dehydrogenase Activity

LDH activity was measured as described in Chapter 2.

4.2.7 Preparation of Whole Cell Lysates

Whole cell lysates were prepared as described in Chapter 2.

4.2.8 Western Blots

Western blotting was performed as outlined in Chapter 2. 15μg of lysate protein were run for all samples. Membranes were incubated overnight at 4°C with an antibody to MnSOD (1:5000 dilution), CuZnSOD (1:1000 dilution), hsp70 (1:1000 dilution), hsp60 (1:2000 dilution) or mtTFAM (1:250 dilution). The membranes were visualized using the Odyssey infrared imaging system from LI-COR Biosciences, with IR-linked secondary antibodies to rabbit or mouse (1:5000 dilution). Western blot analysis was performed using Odyssey imaging software 1.0.

4.2.9 Citrate Synthase Activity

Citrate synthase (CS) activity was measured as in Chapter 2.

4.2.10 Antioxidant Enzyme Activities

Catalase and Glutathione Peroxidase activity was measured as outlined in Chapter 2.

4.2.11 Statistical Analysis:

Data were analyzed by repeated measures ANOVA using Systat v.12. Post-hoc comparisons between means were done by Tukey's test. Data comparing two experimental groups were analyzed using the student's t-test. All data are presented as means ± standard error of the mean (SEM). A p-value of < 0.05 was considered significant.

4.3 Results:

The effects on cell growth of two of the most abundant RES analogues found in blueberries and red wines (piceid and pterostilbene) (Adrian et al., 2000) and two prevalent human metabolites of RES (RES-S and RES-G) (Wenzel and Somoza, 2005) were investigated in C2C12 myoblasts, an ERbeta positive cell line. While neither of the metabolites, RES-S or RES-G, had any effect on myoblast proliferation at concentrations up to 50μM, piceid (50μM) and pterostilbene (20μM) significantly increased the population doubling times of myoblasts (Fig. 4.1).

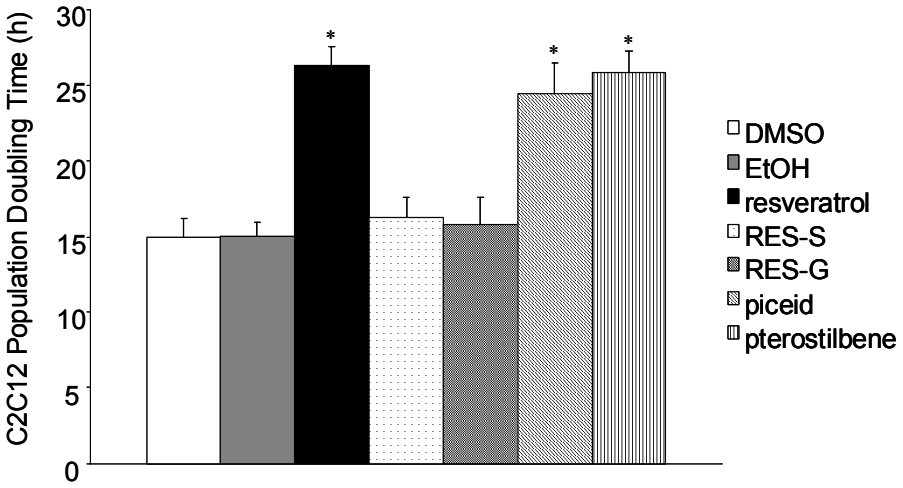


Figure 4.1. Piceid and pterostilbene increase myoblast population doubling time. Average population doubling time in C2C12 myoblasts treated with DMSO, ethanol, resveratrol (25μM) RES-S (50μM), RES-G (50μM), piceid (50μM) and pterostilbene (20μM in ethanol). Data shown represents the means of 4 independent trials. Error bars represent SEM. *=$p<0.05$ compared to vehicle control.

RES's ability to enhance cellular stress resistance may play a key role in its apparent protective properties in the brain and cardiovascular system. Pre-treatment with RES increases the resistance of C2C12 cells to various exogenous stressors (Robb and Stuart, 2011; Chapter 2). It was therefore predicted that pterostilbene and

piceid would similarly impart cytoprotection. The LD_{50} for hydrogen peroxide was measured in cells continuously treated with RES, pterostilbene, piceid, or vehicle control for 48h. Since these compounds are themselves antioxidants, they were removed from the media by refreshing cells with new media 1h prior to exposure to hydrogen peroxide. Both piceid and pterostilbene significantly increased the hydrogen peroxide LD_{50} in myoblasts and fibroblasts (Fig. 4.2).

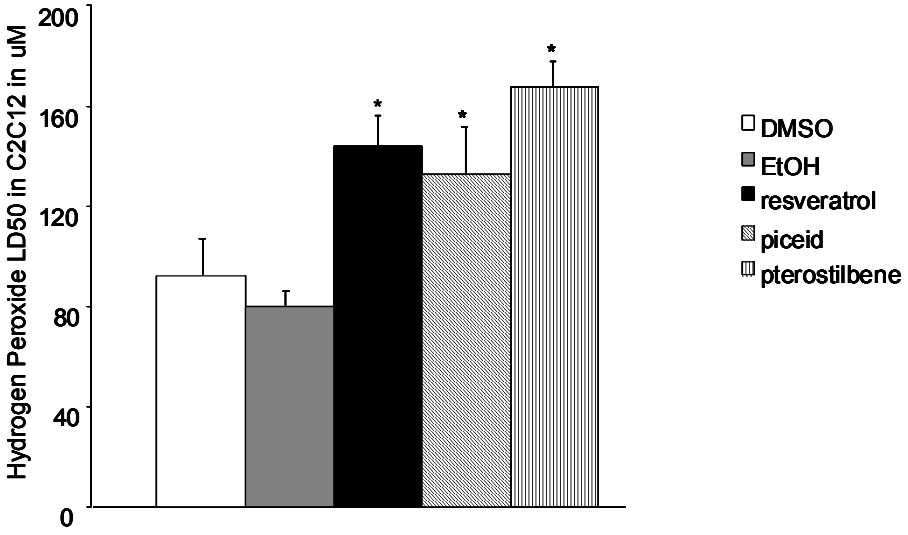


Figure 4.2. Piceid and pterostilbene increase stress resistance in myoblasts. Hydrogen Peroxide LD_{50} in A. C2C12 myoblasts treated with DMSO, ethanol, resveratrol (25μM), piceid (50μM) and pterostilbene (20μM in ethanol). Data shown represents the means of 3-5 independent trials. Error bars represent SEM. *=$p<0.05$ compared to vehicle control.

4.3.2 Effects of Resveratrol Analogues on Stress Resistance Proteins

Induction of the mitochondrial antioxidant enzyme MnSOD expression appears to be a critical event underlying RES's effects on cell growth and cellular stress resistance (Robb and Stuart, 2011; Chapter 2). It was therefore hypothesized that, as structurally related RES analogues, piceid and pterostilbene might similarly induce MnSOD expression. Indeed, both piceid (50μM) and pterostilbene (20μM) significantly increased

MnSOD levels, in agreement with their observed effects on cell proliferative growth and stress resistance. No significant differences in MnSOD protein levels were observed in cells treated with the RES metabolites RES-S (50μM) and RES-G (50μM), again in agreement with their inability to elicit effects on proliferative growth or stress resistance (Fig. 4.3).

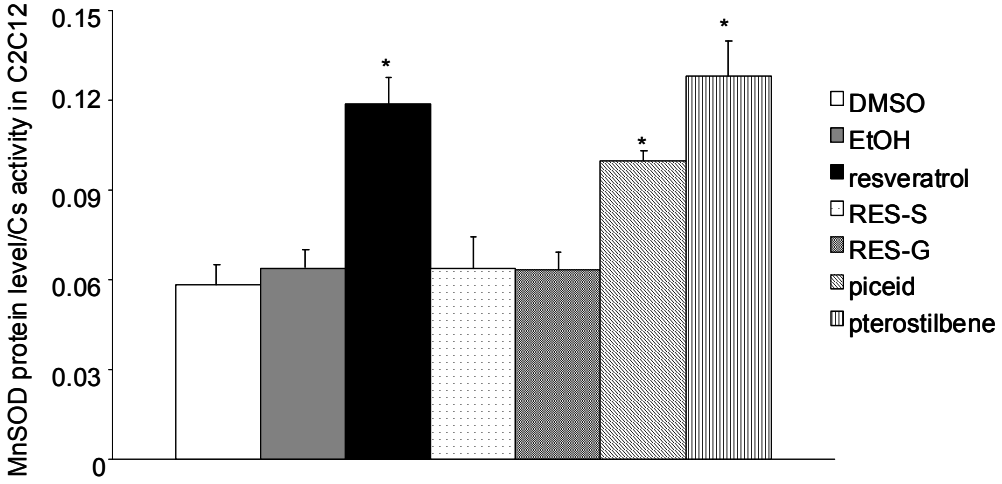


Figure 4.3. Piceid and pterostilbene increase MnSOD protein levels in myoblasts. MnSOD protein levels normalized to citrate synthase activity in C2C12 myoblasts treated with DMSO, ethanol, resveratrol (25μM), RES-S (50μM), RES-G (50μM), piceid (50μM) and pterostilbene (20μM in ethanol). Since phytoestrogens including resveratrol can stimulate a general increase in mitochondrial abundance in many cell types, to accurately evaluate changes in MnSOD levels citrate synthase activity was used as a proxy of mitochondrial abundance. Data shown represents the means of 3 independent trials. Error bars represent SEM. *=$p<0.05$ compared to vehicle control.

Although MnSOD overexpression alone can confer stress resistance in a variety of cell types (St Clair et al., 1991) other mechanisms, including overexpression of antioxidant enzymes such as glutathione peroxidase 1 (Faucher et al., 2003) or CuZnSOD (Chen et al., 2000) can elicit broadly enhanced resistance to cellular oxidative stress. To evaluate if red wine polyphenols were influencing the activities of other antioxidant enzymes, the activities of catalase and glutathione peroxiadse, and the protein level of the

enzyme CuZn superoxide dismutase were measured in treated and control cells. No significant differences in either catalase or gluathione peroxidase activity were found in any of the treatment groups (Table 4.1). Similarly, no significant changes in CuZn superoxide dismutase protein levels were found in any of the experimental groups (Table 4.1).

Heat shock proteins (Hsps) also play key roles in cellular stress resistance (Bukau et al., 2006). Since an upregulation of Hsp expression might partially explain observations of increased stress resistance, the levels of the cytosolic Hsp70, and the mitochondrial Hsp60 were measured in both the polyphenol treated and control cells. No significant changes in the levels of either Hsp60 or Hsp 70 were found in any of the treatment groups (Table 4.1). Thus, piceid and pterostilbene elicited reduced proliferative growth and/or enhanced stress resistance concomitantly with induced MnSOD expression. However, there was no general induction of other antioxidant enzymes or of Hsps.

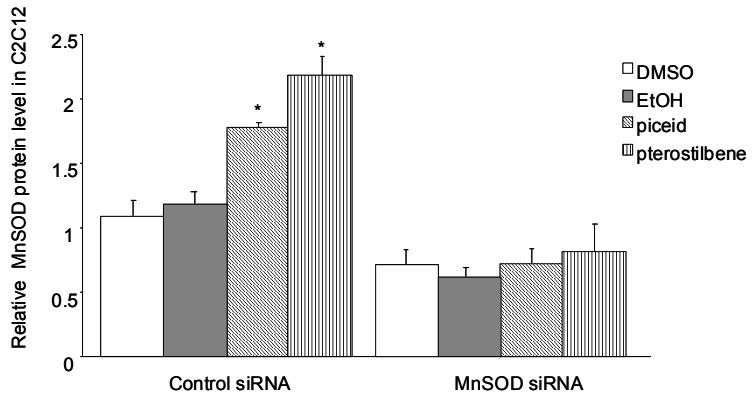
Table 4.1: **Effects of Resveratrol Analogues on Antioxidant Enzymes and Heat Shock Proteins. Catalase activity, Glutathione Peroxidase activity and CuZnSOD, Hsp60 and Hsp 70 protein level in C2C12 myoblasts.**

Treatment	Catalase Activity (mmol/min /mg cellular protein)	Glutathione peroxidase Activity (mmol/min /mg cellular protein	CuZn superoxide dismutase Protein Level Relative to internal control	Hsp60 Protein Level/Cs Activity Relative to internal control	Hsp70 Protein Level Relative to internal control
DMSO	15.51±0.24	67.32±5.27	0.96±0.12	0.089±0.020	1.05±0.22
EtOH	15.80±0.47	66.52±8.14	0.99±0.08	0.097±0.018	0.91±0.36
Resveratrol	15.42±0.07	51.41±5.04	1.25±0.43	0.102±0.010	1.12±0.18
RES-S	N.D.	N.D.	1.06±0.17	0.100±0.015	1.25±0.13
RES-G	N.D.	N.D.	1.11±0.13	0.067±0.011	1.27±0.15
Piceid	19.18±0.50	51.22±3.07	1.26±0.19	0.106±0.018	1.34±0.17
Pterostilbene	16.32±0.34	68.41±7.58	1.20±0.16	0.095±0.014	1.21±0.09

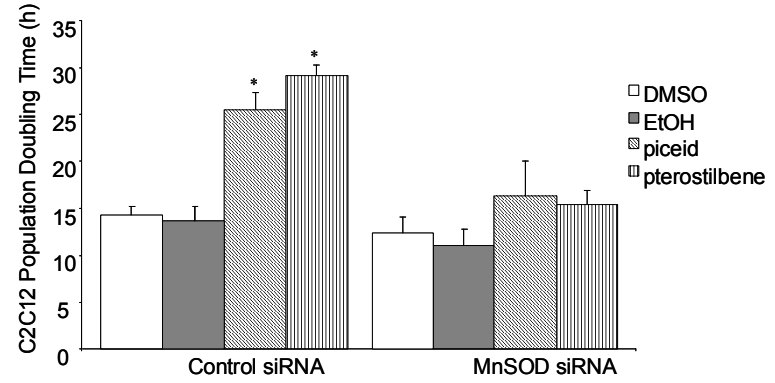
Data represents the mean of duplicate measurements for 3 independent trials. $*= p<0.05$ compared to vehicle control. N.D.= not determined

To investigate the role of MnSOD in the cell growth and stress resistance changes affected by piceid and ptersotilbene, siRNA against MnSOD was used to prevent its increase (Fig. 4.4A). When the increased MnSOD expression elicited by polyphenols was prevented by siRNA, the ability of either piceid or ptersostilbene to inhibit myoblast proliferative growth was abolished (Fig. 4.4B). Similarly, when MnSOD induction was prevented using this siRNA approach, piceid and pterostilbene had no effect on myoblast stress resistance (Fig. 4.4C).

4.4 A



4.4 B



4.4 C

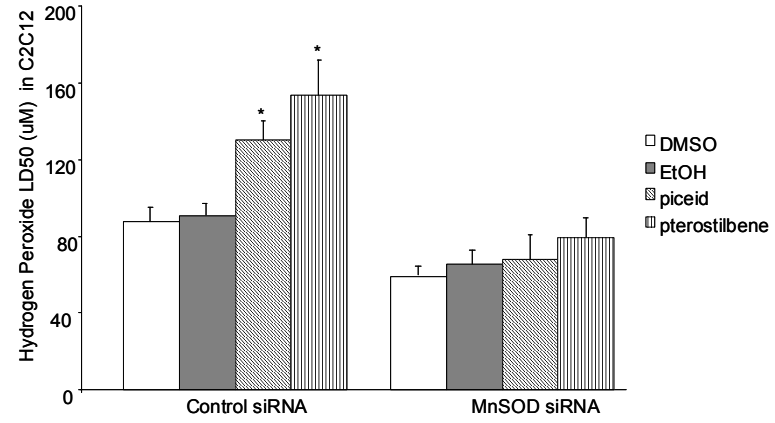


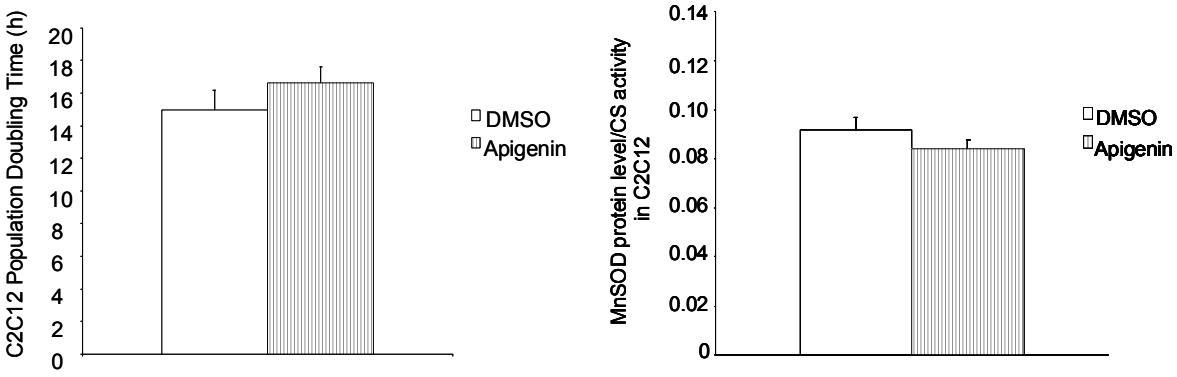
Figure 4.4. MnSOD is essential for piceid and pterostilbene to increase population doubling time and stress resistance in C2C12.
A. MnSOD protein level in C2C12 myoblasts treated with control siRNA or MnSOD siRNA, B. Average population doubling time in C2C12 myoblasts treated with control siRNA or MnSOD siRNA, C. Hydrogen Peroxide LD_{50} of C2C12 myoblasts treated with control siRNA or MnSOD siRNA ± DMSO, ethanol, piceid (50μM) or pterostilbene (20μM in ethanol). Data shown represents the mean of 3 independent trials. Error bars represent SEM. *=$p<0.05$ compared to vehicle control.

The above results suggest a central role for MnSOD in these phytoalexins' effects. However, a relatively wide range of other mechanisms with the potential to act upstream of the MnSOD induction have been proposed to explain RES's biological activities, so the possible contribution of several of these to the inhibition of cell growth and MnSOD levels was investigated. Since the effects of RES and some other phytoestrogens have been suggested to arise from their activities as tyrosine kinase inhibitors (Agullo et al., 1997), the effects of a known tyrosine kinase inhibitor (apigenin) on growth (Fig. 4.5A) and MnSOD induction (Fig. 4.5A) were evaluated. No significant effect of apigenin (up to 75μM) on either cell growth or MnSOD protein levels was observed, indicating that these effects cannot be duplicated by a tyrosine kinase inhibitor.

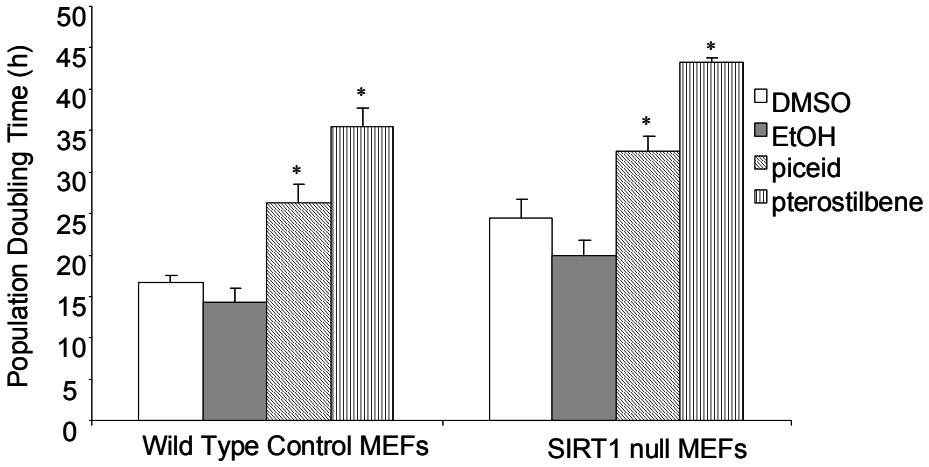
Stimulation of SIRT1 activity is also commonly cited as a molecular mechanism underlying some of RES's biological effects. The effects of both piceid and pterostilbene on cell growth persisted in cells lacking SIRT1. Pterostilbene significantly increased MnSOD protein levels in the SIRT1 null fibroblasts, though this trend did not reach statistical significance for piceid. These data do not support the hypothesis that a direct stimulation of SIRT1 was not necessary for the effects of piceid and pterostilbene on cell growth and MnSOD expression.

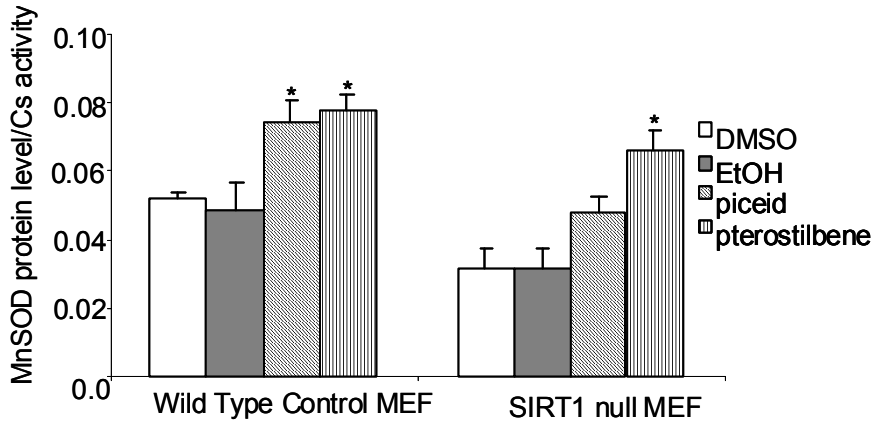
Recently it was reported that certain *in vivo* effects of RES could be attributed to its inhibition of cAMP phosphodiesterases (Park et al., 2012). To determine whether this mechanism contributed to my observations the effect of the cAMP phosphodiesterase inhibitor Rolipram on cell growth and MnSOD induction was evaluated. Rolipram (up to 100μM) had no significant effect on population doubling time or MnSOD protein levels in C2C12 myoblasts.

4.5 A


C2C12 Population Doubling Time (h)
20
18
16
14
12
10
8
6
4
2
0
DMSO
Apigenin
MnSOD protein level/CS activity in C2C12
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
DMSO
Apigenin

4.5 B


Population Doubling Time (h)
50
45
40
35
30
25
20
15
10
5
0
*
*
*
*
DMSO
EtOH
piceid
pterostilbene
Wild Type Control MEFs
SIRT1 null MEFs


MnSOD protein level/Cs activity
0.10
0.08
0.06
0.04
0.02
0.0
*
*
*
DMSO
EtOH
piceid
pterostilbene
Wild Type Control MEF
SIRT1 null MEF

4.5 C

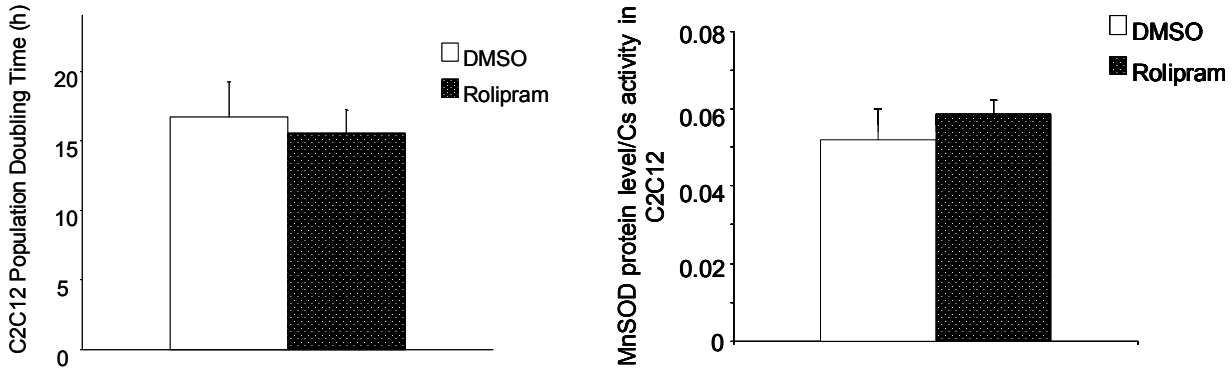


Figure 4.5. Resveratrol, piceid and pterostilbene effects on population doubling time and stress resistance are not due to tyrosine kinase inhibition, SIRT1 activation, and can not be replicated by phosphodiesterase inhibition.
A. Average population doubling time and MnSOD protein levels in C2C12 myoblasts treated with vehicle control or apigenin (75μM). B. Population doubling time, MnSOD protein levels in SIRT1 control and SIRT1 null MEFs treated with DMSO, ethanol, piceid (50μM) or pterostilbene (20μM in ethanol). C. Average population doubling time, MnSOD protein level in C2C12 myoblasts treated with DMSO or Rolipram (50 μM). Data shown represents the mean of 3 independent trials. Error bars represent SEM. *=$p<0.05$ compared to vehicle control.

RES is an ER agonist (Gehm et al., 1997; Bowers et al., 2000). RES's effects on MnSOD expression, cell proliferation and stress resistance are abolished by an ER antagonist and can be reproduced using the ERbeta agonist DPN (Robb and Stuart, 2011; Chapter 2). To determine if the observed effects of piceid and pterostilbene were also being mediated through ERs, the treatments with these polyphenols were repeated in the presence of estrogen antagonist ICI182780. Co-treatment with ICI182780 abolished the effects of both piceid and pterostilbene on population doubling time, resistance to hydrogen peroxide induced cell death and MnSOD protein levels in C2C12 myoblasts (Table 4.2). This suggests that, similar to RES, the mechanisms of piceid and pterostilbene involved signaling through ERs.

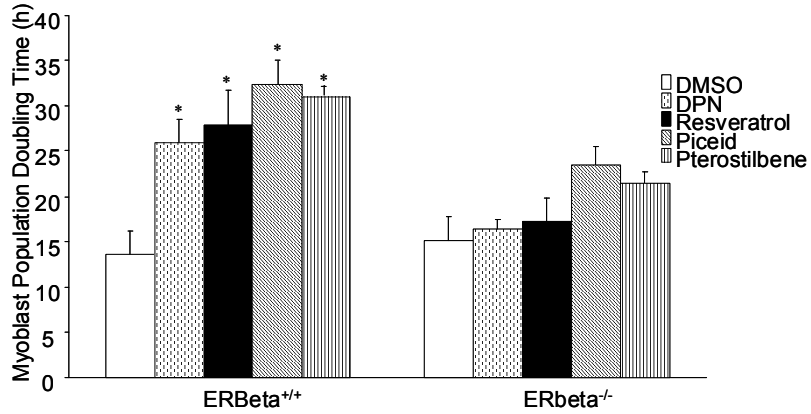
Table 4.2: **Effects of Resveratrol Analogues on Population Doubling Time, Resistance to Hydrogen Peroxide Induced Cell Death, and MnSOD Protein Level in the Absence and Presence of ICI182780 in C2C12 myoblasts.**

Treatment	Population Doubling Time (h)		H_2O_2 LD_{50} (μM)		MnSOD Protein Level Relative to Internal Control	
	No Antagonist	Antagonist	No Antagonist	Antagonist	No Antagonist	Antagonist
DMSO	14.98±1.16	15.29±0.11	92±11	82±7	1.06±0.15	0.94±0.12
EtOH	15.01±0.94	14.87±0.16	84±9	93±6	1.10±0.18	1.04±0.08
Resveratrol	26.34±1.25∗	17.23±1.22	144±19∗	96±11	2.10±0.24∗	0.82±0.16
Piceid	27.14±1.11∗	21.24±1.05	149±16∗	89±17	1.84±0.17∗	0.97±0.10
Pterostilbene	33.61±1.08∗	22.19±2.17	166±18∗	110±9	2.22±0.29∗	1.19±0.18

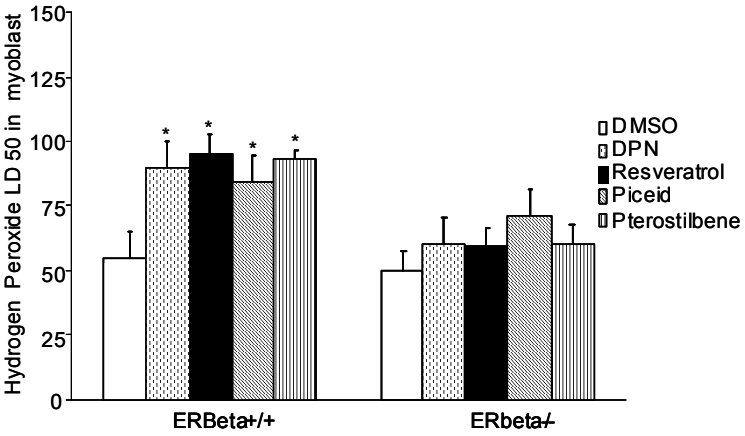
Antagonist = ICI182780. Data represents the mean of duplicate measurements for 3 independent trials. ∗= $p<0.05$ when compared to vehicle control.

Estrogen signaling is predominately mediated by two receptors: ERalpha and ERbeta. To further determine the specific role of ERbeta in mediating the effects of RES and other phytoestrogens, myoblasts from ERbeta null mice were used. The absence of ERbeta abolished the effects of DPN (a well characterized ERbeta agonist used here as a positive control), RES, piceid and pterostilbene on population doubling time and MnSOD protein levels (Fig 4.6A). Similarly, DPN, RES, piceid and pterostilbene had no effect on stress resistance of ERbeta null cells (Fig 4.6B). Thus ERbeta is required for the effects of RES, piceid and pterostilbene studied here.

4.6 A



4.6 B



4.6 C

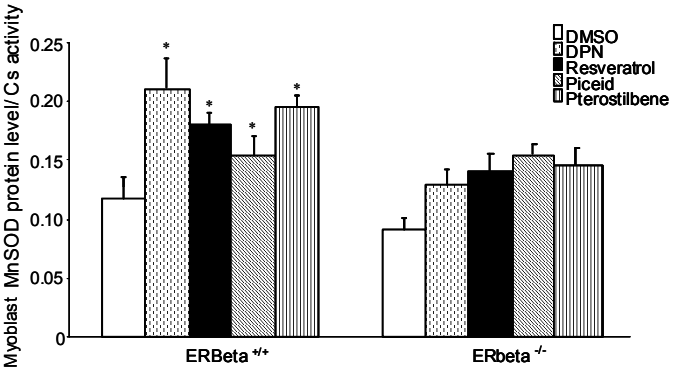
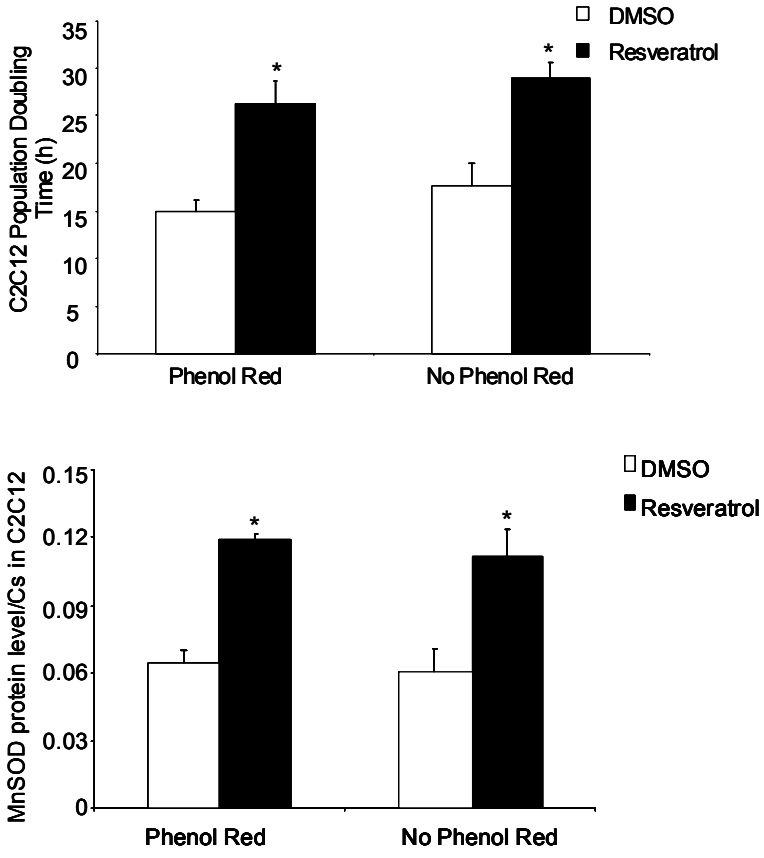


Figure 4.6. ERbeta is involved in the effects of resveratrol, piceid and pterostilbene. A. Average population doubling time, B. Hydrogen Peroxide LD_{50}, C. Average MnSOD protein levels normalized to citrate synthase activity in control or ERbeta null myoblasts treated with DMSO, DPN (10μM), resveratrol (25μM), piceid (50μM) and pterostilbene (10μM). Data shown represents the mean of 5 independent trials. Error bars represent SEM. *=$p<0.05$ compared to vehicle control.

The phenol red contained in many cell culture media, and the various hormones and growth factors found in fetal bovine serum have the potential to influence the cellular effects of phytoestrogens in vitro. To determine if these factors might have contributed to my observations, the effects of RES were evaluated in the presence and absence of phenol red, and in culture medium containing standard and charcoal stripped fetal bovine serum. In both instances the increase in C2C12 population doubling time and MnSOD protein levels observed with RES treatment persisted. From these results it is clear that neither phenol red nor interactions with fetal bovine serum were contributing to the observed effects.

4.7 A



4.7 B

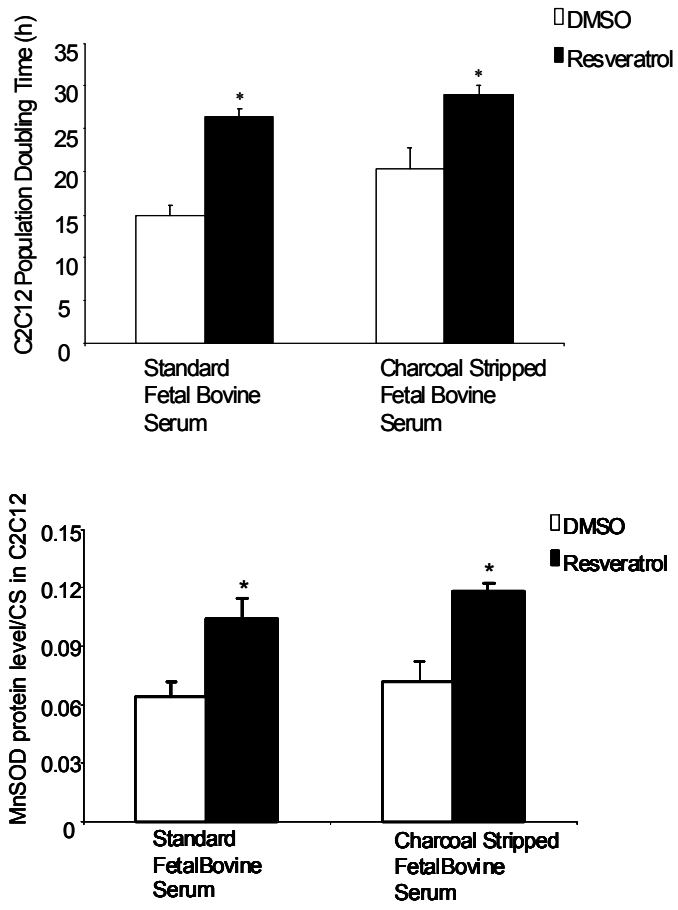
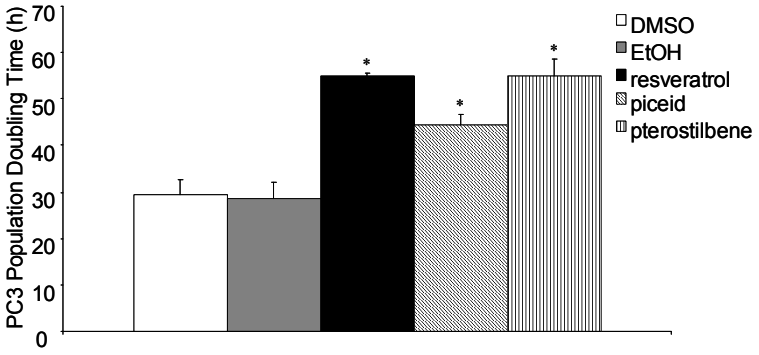


Figure 4.7. Resveratrol's effects on population doubling time and MnSOD are not affected by phenol red or standard fetal bovine serum.
A. Average population doubling time and MnSOD protein levels of C2C12 myoblasts treated with DMSO or resveratrol in medium with and without phenol red B. Average population doubling time and MnSOD protein levels of C2C12 myoblasts treated with DMSO or resveratrol in medium containing standard fetal bovine serum or charcoal stripped fetal bovine serum Data shown represents the mean of 3 independent trials. Error bars represent SEM. *=$p<0.05$ compared to vehicle control.

RES has been extensively investigated for its potential to slow the growth of cancerous cells. Recently piceid, pterostilbene and other red wine polyphenols have been investigated in this context. To determine whether RES, piceid, and pterostilbene elicited similar effects on cell growth and MnSOD induction in cancerous cells, their effects were

measured in ERbeta expressing prostate cancer cell line PC3. As in C2C12 myoblasts and primary mouse myoblasts all of these compounds slowed the growth of PC-3 cells concomitant with a significant induction of MnSOD protein expression.

4.8 A



4.8 B

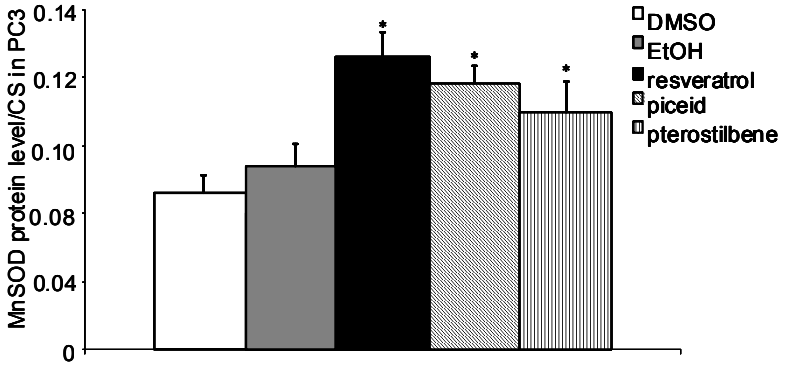
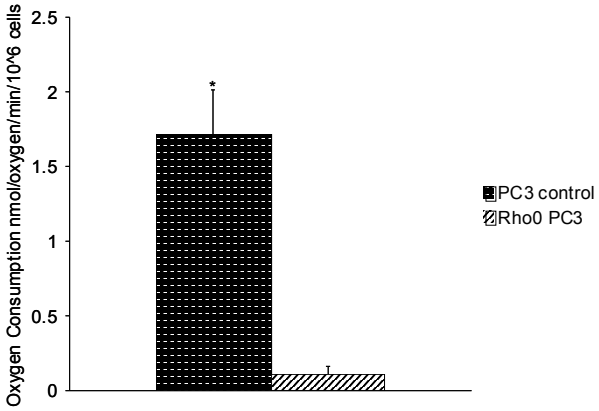


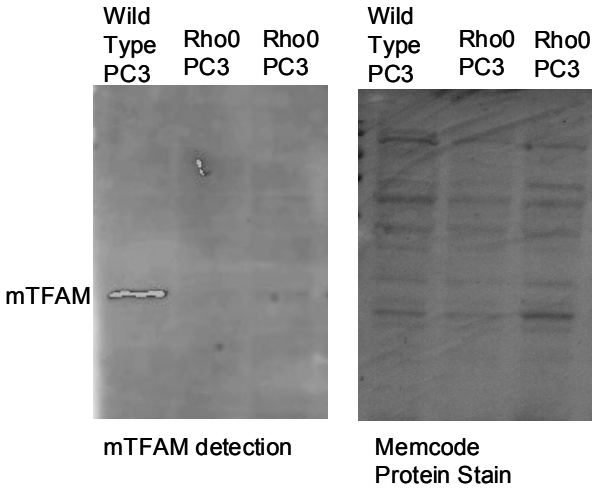
Figure 4.8. Resveratrol, piceid and pterostilbene increase PC3 population doubling time and MnSOD protein levels.
A. Average population doubling time and B. MnSOD protein levels in PC3 cells treated with DMSO, ethanol, resveratrol (25μM), RES-G (50μM), piceid (50μM) and pterostilbene (20μM in ethanol). Data shown represents the means of 4 independent trials. Error bars represent SEM. *=$p<0.05$ compared to vehicle control.

The only enzymatic activity ascribed to MnSOD is it role in mitochondrial redox metabolism catalyzing the dismutation of superoxide to produce hydrogen peroxide. To specifically investigate the role of mitochondrial respiratory ROS in the changes in proliferative cell growth associated with RES treatment, experiments were repeated in

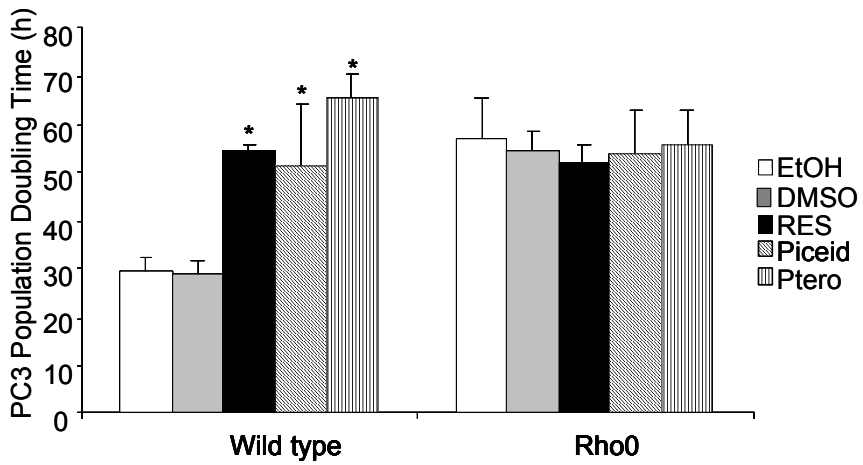
rho0 PC3 cells, which do not respire (Fig. 4.9A) and therefore produce low levels of mitochondrial ROS (Hoffman et al 2004). Interestingly, RES induced MnSOD in these cells to virtually the same extent as in normal PC3 cells (Fig. 4.9B) but had no effect on proliferative growth (Fig. 4.9C). This suggests that the ROS metabolizing activity of MnSOD is necessary for its influence on replicative cell growth.

4.9A





4.9B



4.9C

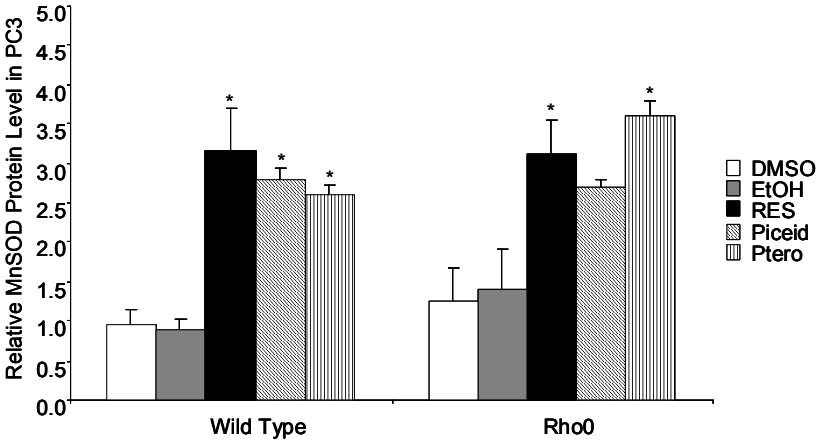


Figure 4.9. RES affects MnSOD, but not population doubling time in the absence of mitochondrial respiration.
A. Oxygen consumption and mtTFAM detection in control and rho0 PC3 cells B. MnSOD protein levels in control and rho0 PC3 cells treated with DMSO or 20μM RES for 48h. C. Population doubling time in control and rho0 PC3 cells treated with DMSO or 20μM RES for 48h. n=3. Error bars represent SEM. *=$p<0.05$ compared to vehicle control.

4.4 Discussion:

Two of the most abundant metabolites of RES *in vivo*, RES-S and RES-G (Wenzel et al., 2005), did not affect myoblast proliferation and stress resistance *in vitro*. In agreement with these results, proliferation of the cancerous mammary cell line MCF7 is unaffected by the 3'-sulfate and 4-sulfate RES metabolites (Miksits et al., 2009). In contrast, sulfate metabolites do have shared biological activity with RES in cultured macrophage cells, in which the 3'-sulfate, 3,4'-sulfate and 3-sulfate metabolites significantly inhibit the activity of inducible nitric oxide synthase (Hoshino et al., 2010). Thus, RES metabolites may have some overlapping biological activities with RES that extend beyond the measurements made in this study. Evaluating the biological activity of RES metabolites in different experimental contexts is an important goal that will be achieved as commercial sources for these compounds become increasingly available.

Two compounds closely related to RES, piceid and pterostilbene, decreased the rates of myoblast growth and increased cellular stress resistance *in vitro*. In addition, all three polyphenols significantly reduced proliferative growth in the PC3 prostate cancer cell line. This observation is in agreement with existing reports that pterostilbene inhibits the growth of cancerous breast (MCF7; MDA-MB-231), colon (HCT116, HT29, Caco-2) and prostate cell lines (PC3, LNCaP) (Moon et al., 2012; Mannal et al., 2010; Nutakul et al., 2011; Lin et al., 2009). It is unlikely that the striking overlap between the cellular effects of RES and pterostilbene can be explained by a conversion of these compounds to RES *in vitro*. In mice given an oral dose of 200mg/kg pterostilbene, RES was not detected in a screen of urinary metabolites, which suggests that it is not a product of pterostilbene metabolism (Shao et al., 2010).

Although experiments *in vitro* are necessary for examining detailed molecular mechanisms of action, a major criticism of this research has been that the activities of RES and related compounds *in vitro* are only observed at supraphysiological concentrations that cannot be achieved *in vivo*. Indeed, it is surprising that, while concentrations above 10μM are required to elicit effects *in vitro*, similar effects are typically observed *in vivo* even though measured plasma levels never exceed 1-2μM. This apparent discrepancy is likely explained by the fact that, although RES (or related molecules) are added *in vitro* to concentrations of 10μM and greater, their extensive binding to serum proteins present in most media containing 10% fetal calf serum greatly lowers their effective free concentrations. In cell-free culture medium the percentage of free RES falls to below 10% of the initial dosage within ten hours due to its interaction with serum factors (Jannin et al., 2004). Uptake of [^{3}H]-RES in human colon adenocarcinmoa cells is significantly reduced when RES is added to growth medium containing fetal calf serum, again apparently due to the binding of RES to serum constituents (Colin 2011). Thus, the free concentrations of RES, pterostilbene and piceid used in most *in vitro* studies, including the present study, are likely to be appreciably reduced by serum protein binding.

The anticancer properties of RES and pterostilbene have been reported in a number of cell types and in animal models (Baur and Sinclair, 2006; Kostin et al., 2012; McCormack et al., 2012; Moon et al., 2012). The identification of MnSOD as an essential target of piceid and pterostilbene is interesting given the accumulating evidence that MnSOD can regulate cell cycle progression and proliferative growth (Sarsour et al., 2008), and that MnSOD overexpression alone significantly reduces

proliferation in transformed human fibroblasts, malignant pancreatic and prostate cell lines (Yan et al., Weydert et al., 2003; Li et al., 1999). Very recent evidence indicates that MnSOD is upregulated in pancreatic cancer (MIAPaCa-2, PANC-1) and breast cancer (MCF7, MDA-MB231) cell lines treated with pterostilbene (Kostin et al., 2012; McCormack et al., 2012; Moon et al., 2012). Thus, the induction of MnSOD elicited by RES, piceid and pterostilbene in cancerous cell lines may provide a shared mechanism responsible for the anticancer properties of these molecules.

In the context of cellular stress resistance, MnSOD overexpression in pheochromocytoma PC6 cells protects against apoptosis induced by excess iron, the β-amyloid peptide and nitric oxide producing compounds (Keller et al., 1998). The only enzymatic activity ascribed to MnSOD is the dismutation of superoxide anions to hydrogen peroxide, and it is therefore probable that its effects occur as a result of a change in the intracellular (or intramitochondrial) redox environment. MnSOD produces hydrogen peroxide as a product of superoxide dismutation, and this hydrogen peroxide may act as an intracellular signaling molecule via the oxidation of critical cysteine residues involved in cell cycle progression (Holley et al., 2011). A second reaction modified by MnSOD is that between the superoxide anion and nitric oxide to produce the highly reactive peroxynitrite molecule. Overexpression of MnSOD reduces the incidence of nitrosylated tyrosine residues in mitochondrial complexes and mtDNA damage following ethanol administration in mice, while partial deletion of MnSOD exacerbates peroxynitrite mediated damage (Larosche et al., 2010). By reducing the availability of the superoxide anion MnSOD can in turn reduce the production of the peroxynitrite, which may be important to cellular stress resistance.

The molecular mechanism responsible for the decrease in cell proliferation and increase in MnSOD in response to RES, pterostilbene and piceid treatment in myoblasts was not due to their actions as tyrosine kinase inhibitors, SIRT1 activators or phosphodiesterase inhibitors. Apigenin, a polyphenol marketed as a tyrosine kinase inhibitor (Agullo et al., 1997), did not replicate RES's effects on proliferation or MnSOD levels, suggesting that this activity does not mediate RES's *in vitro* effects. Acetylation and deacetylation are increasingly recognized for their roles in the regulation of cell function, including important intracellular processes such as the cellular stress response (ie. FOXO transcription factors, heat shock protein factor 1), and cell metabolism (i.e. PGC1-alpha) (Burnett et al., 2011; Westerheide et al., 2009; Nemoto et al., 2005). In mammals deletion of SIRT1 is extremely harmful, resulting in metabolic dysregulation and increased incidence of autoimmune disease (Seifert et al., 2012; Sequeira et al., 2008). It was observed that the SIRT1 deletion substantially reduced the activity of the Krebbs cycle enzyme citrate synthase (data not shown). However, the ability of piceid and pterostilbene to slow cell division and increase MnSOD levels did not require SIRT1. An increase in MnSOD levels in the brains of the Alzheimer's disease model SAMP8 mouse also occurred without an increase SIRT1 activity (Chang et al., 2011). Thus, the effects of pterostilbene and piceid on MnSOD, and in turn cell proliferation do not require activation of SIRT1 activity.

Recently RES was reported to elicit effects on C2C12 myotubes via an inhibition of cAMP phosphodiesterases (Park et al., 2012). The phosphodiesterase inhibitor Rolipram had no effect on proliferation or MnSOD protein levels in replicating C2C12 myoblasts. Interestingly, in differentiated C2C12 myotubes RES does not elicit an

increase in MnSOD protein levels (data not shown). It may be the case that RES's induction of MnSOD is unique to replicating cell types, and that other mechanisms, such as the inhibition of phosphodiesterase, play a more central role in its effects in differentiated cells.

Polyphenols including RES, can react with constituents of culture media (*i.e.* phenol red), and can undergo redox cycling reactions to increase ROS concentrations (Halliwell, 2008). Phenol red can also act as an estrogen mimetic in culture (Berthois et al., 1986). The effects of RES on proliferation and MnSOD persisted in the absence of phenol red, suggesting that they were not a response to increased ROS due to redox cycling, or the estrogenic effects of phenol red. These results were likewise unaffected by the use of charcoal stripped fetal bovine serum, suggesting they were not dependent upon hormonal factors present in the culture media.

In the absence of oxidative metabolism, RES, piceid and pterostilbene failed to significantly affect population doubling time in prostate cancer cells. This observation provides experimental evidence in support of the hypothesis that the antiproliferative effects of MnSOD upregulation are directly related to its effects on intracellular redox status. The redox sensitive targets responsibe for the effects on cell cycle progression were not determined here.

An estrogen antagonist inhibited the effects of pterostilbene and piceid on proliferation, stress resistance and MnSOD, suggesting that estrogen signaling is an essential component of their biological activities. Estrogen treatment alone increases MnSOD protein levels in rat aortic vascular smooth muscle cells, MCF-7 (a cancerous mammary cell line) and mouse myoblasts, and the induction of MnSOD is essential for

its antiproliferative effects on vascular smooth muscle cells (Borrás et al., 2005; Sivritas et al., 2011). Estrogen signaling is mainly mediated by ERalpha and ERbeta, and the C2C12 cell line used here expresses both estrogen receptor forms (Milanesi et al., 2008). ERbeta reduces proliferative cell growth under a variety of experimental conditions. Treatment of the colon cancer cell line MC38 with the specific ERbeta agonist DPN significantly reduces cell proliferation (Motylewska et al., 2009). *In vivo* DPN administered to Apc(Min/+) mice significantly reduced their incidence of intestinal tumors (Giroux et al., 2011). Lentivirus transfection of ERbeta into a cancerous colon cell line significantly reduces cell proliferation (Hartman et al., 2009).

A recent computation study predicted that RES binds preferentially to ERbeta over ERalpha (Yuan et al., 2011). In agreement with the importance of ERbeta in RES's molecular mechanism, the effects of DPN, RES, pterostilbene and piceid on proliferation, stress resistance and MnSOD were absent in cell lines generated from ERbeta null mice. This observation indicates a shared molecular mechanism for RES, piceid and pterostilbene.

In conclusion, pterostilbene and piceid are capable of eliciting a reduction in proliferative growth and confer enhanced stress resistance through a mechanism that requires an induction of MnSOD and the presence of ERbeta. This detailed knowledge of intracellular mechanisms underlying the biological activities of some phytoestrogens will be important in directing their continuing development as nutraceuticals. In addition to being effective at lower concentrations in this study, pterostilbene also has a greater bioavailability and cellular uptake than RES (Nutakul et al., 2011; Lin et al., 2009).

Given the similarities in cellular effects and mechanism, pterostilbene may be a promising alternative to RES for nutritional and medicinal purposes.

Chapter 5: A Shared Molecular Mechanism for the Effects of Phytoestrogens on Proliferation, Stress Resistance and Antioxidant Enzymes

Hypothesis: MnSOD activity is capable of reducing proliferative cell growth and imparting cytoprotection. Given that treatment with the phytoestrogen RES can elicit an induction of MnSOD via an interaction with ERbeta, it is hypothesized that other phytoestrogens capable of acting as ERbeta agonists will similarly induce MnSOD and in turn reduce rates of proliferative cell growth and confer cytoprotection.

Objectives: The objectives of this project were: **1)** to examine the ability of seven phytoestrogens to affect cell proliferation, stress resistance and antioxidant enzymes, **2)** to study the role of ERbeta in the actions of these phytoestrogens.

Publications of results:

Robb EL, Stuart JA. Multiple phytoestrogens inhibit cell growth and confer cytoprotection by inducing manganese superoxide dismutase expression. Phytotherapy Research, *In Press,* 2013.

Contributions:

I performed all experiments, statistical analysis and assisted in the manuscript preparation

5.1 Introduction:

The antioxidant enzyme Manganese superoxide dismutase (MnSOD) is the sole superoxide dismutase in the mitochondrial matrix, where it catalyzes the conversion of superoxide produced primarily by respiratory complexes into hydrogen peroxide. MnSOD plays a critical role in the metabolism of mitochondrial reactive oxygen species. $MnSOD^{-/-}$ mice die either in utero or soon after birth (Lebovitz et al., 1996; Li et al., 1995). On the other hand, transgenic overexpression of MnSOD is neuroprotective (*e.g.* Shan et al., 2007; Dumont et al., 2009), cardioprotective (Shen et al., 2006), and confers protection against a wide range of cellular stresses including radiation (Hosoki et al., 2012) and diabetes mellitus (Boden et al., 2012).

In addition to its cytoprotective function, MnSOD also plays a key regulatory role in the cell cycle, although the exact mechanism(s) involved are just beginning to be elucidated (Sarsour et al., 2012). Early observations indicated that a high proportion of cancerous cells had lower than normal MnSOD levels, and that when these were restored by transgenic MnSOD expression the rate of proliferative growth was slowed markedly (Li et al., 1995). This has now been demonstrated in a number of cancer cell lines (Li et al., 1998; Li et al., 1999; Weydert et al., Ough et al., 2004), and the same effect has been shown also in non-transformed cells, both *in vitro* (Zhang et al., 2010) and *in vi*vo (Kim et al., 2010).

A significant induction of MnSOD expression (2-4 fold) can be achieved using the phytoestrogen RES under both *in vitro* (Robb et al., 2008) and *in vivo* (Robb et al., 2008b; Chapter 6) conditions. The RES-induced upregulation of of MnSOD in fibroblasts, myoblasts, neuroblastoma cells and prostate cancer (PC3) is coincident

with enhanced cellular resistance against paraquat, hydrogen peroxide or methane methylsulfonate (MMS) and reduced proliferative growth rates (Robb and Stuart 2011; Chapter 2). RES is an agonist of both ERalpha and ERbeta (Gehm et al., 1997, Bowers et al., 2000); however its effects on stress resistance and proliferation could be replicated by the specific ERbeta agonist DPN but not the ERalpha agonist PPT (Robb and Stuart 2011; Chapter 2). In addition, RES had no effect on MnSOD expression, cellular stress resistance or proliferative growth in primary myoblasts from ERbeta null mice (Chapter 4). Thus, the induction of MnSOD expression by RES appears to be related to its activity as an ERbeta agonist.

Estrogen is broadly protective in human health, playing an important role in the cardiovascular, skeletal and nervous systems (reviewed in Spence and Voskuhl, 2012). Phytoestrogens are compounds produced by plants that have similar structural attributes to estrogens, and are capable of binding to ERs with a lower affinity and initiating select transcriptional events (Lorand et al., 2010). Dietary phytoestrogens may provide an alternative means to capture estrogen's beneficial effects while circumventing some of the risks associated with maintaining high estrogen levels (Patisaul and Jefferson, 2010). The available data supports the idea that phytoestrogens are capable of replicating estrogen's protective effects, as the overlap in the physiological effects elicited by estrogen and phytoestrogens is striking. For example, similar to what is observed with estrogen treatment, micromolar concentrations of the phytoestrogen genistein protect cultured rat cortical neurons from amyloid beta toxicity (Valles et al., 2008). In a rat model of Alzheimer's disease genistein reduces levels of oxidative damage that are associated with the disease progression (Bagheri et al., 2011). The parallels between

estrogen and phytoestrogens are not restricted to their neuroprotective properties. Similar to estrogen, phytoestrogens are cardioprotective, and can reduce oxidative damage in vascular tissues that is characteristic of diseases such as atherosclerosis (Cassidy et al., 2003).

An important difference between the effects of estrogen and phytoestrogens is that the latter are generally antiproliferative. Genistein inhibits cell cycle progression in cancerous cell lines including HeLa (Kim et al., 2009), the PC-3 prostate cell line (Lee et al., 2012), and ERbeta positive breast cancer cells (Rajah et al., 2009). The phytoestrogens genistein, glycitein and RES significantly reduce proliferative cell growth in the MCF-7 breast cancer cell line (Sakamoto et al., 2010).

The antiproliferative actions of phytoestrogens are often studied in the context of cancer; nevertheless these compounds also impact upon the growth of non-cancerous cell types. In rats, genistein reduces cell proliferation in the cerebral cortex, and apigenin inhibits proliferation of the B104 rat neuronal cell line (Yakisich et al., 1998; Sato et al., 1994). Phytoestrogens are also antiproliferative in muscle satellite cells, which are known to express ERs (Wiik et al., 2009). For example, genistein significantly inhibits the growth of rat aortic smooth muscle cells in culture (Yu et al., 2009). In skeletal myoblasts isolated from neonatal pigs both genistein and daidzein reduce rates of proliferative cell growth (Mau et al., 2008). Thus a reduction in proliferative cell growth is a frequent observation made with phytoestrogen treatment.

Increasing MnSOD protein levels may be an important mechanism mediated by ERbeta that is shared between RES and other phytoestrogens that renders cells slow growing and stress resistant. Phytoestrogens are produced by scores of different plants

and as such are found in many foods and beverages. They encompass a diverse group of compounds including the isoflavones, flavones, coumestrans, flavanoids and stilbenes. In this study eight phytoestrogens belonging to those five groups with predicted estrogenic activity were screened for their effects on proliferative growth rate, cellular stress resistance and the intracellular enzymes that can impart these qualities in cultured myoblasts. Genistein, daidzein and glycitin are isoflavones that are found naturally in legumes with particularly high levels in soy. Apigenin is a flavone found in chamomile, parlsey, celery, apples, and other foods (Patel et al., 2007). Kaempferol and isoliquirtigenin are flavanoids found in many different foods including onions, tea and broccoli or in liquorice, respectively (Calderon-Montano et al., 2011; Tamir et al., 2001). RES is a stilbene found in grapes, peanuts and wine (Soleas et al., 1997). Lastly, coumestrol is a coumestran found in alfalfa sprouts. It is hypothesized that these phytoestrogens will behave similarly to RES, eliciting a reduction in proliferative cell growth and an increase in cellular stress resistance in myoblasts that is concurrent with an induction of MnSOD. It is further hypothesized that ERbeta plays an important role in these observations, and that the phytoestrogen effects will be absent in myoblasts isolated from ERbeta null mice.

5.2 Experimental Procedures

5.2.1 Materials:

trans-RES was obtained from A.G. Scientific (San Diego, CA). Daidzein and Isoliquirtigenin were obtained from Cayman Chemical (Ann Arbor, MI). Genistein and Glycitein were obtained from LC Laboratories (Woburn, MA). Kaempferol and Apigenin were purchased from Tocris Biosciences (Ellisville, MO). All polyphenols purchased were of greater than 95% purity. Their chemical structures are shown in Figure 5.1.

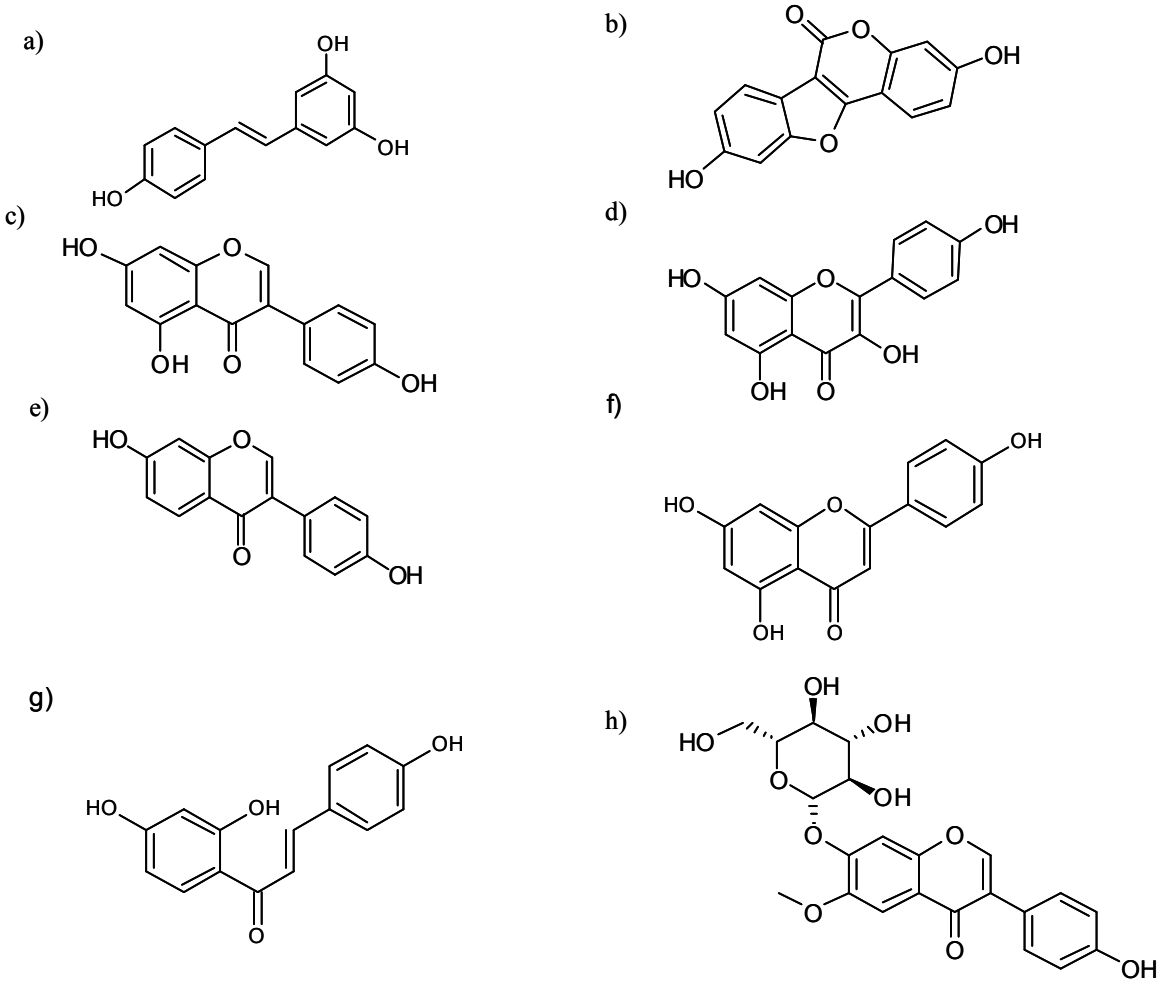


Figure 5.1. Chemical structures of the eight phytoestrogens investigated.
a) resveratrol b) coumestrol c) kaempferol d) genistein e) daidzein f) apigenin g) isoliquirtigenin h) glycitin

ICI182780, DPN and PPT were purchased from Tocris Biosciences (Ellisville, MO). Dulbecco's Modified Eagle Medium with high glucose, Ham's F10 nutrient mixture, penicillin/streptomycin, non-essential amino acids, trypsin and fetal bovine serum were obtained from Hyclone (Logan, UT). Gentamicin, amphotericin B, collagen, pronase and collagenase were obtained from Sigma–Aldrich (St. Louis, MO). BioRad protein dye was purchased from BioRad laboratories (Hercules, CA). Prestained broad range protein marker was purchased from Frogga Bioscience (Toronto, Canada). Pierce Memcode Reversible Protein Stain Kit^{TM} was obtained from Thermo Fisher Scientific (Mississauga, Canada). MnSOD antibody was purchased from Enzo Life Sciences (Brockville, Canada). CuZnSOD antibody was purchased from Stressgen (Victoria, Canada). Antibody to mouse hsp70 was purchased from Stressgen (Victoria, Canada). Hsp 60 antibody was purchased from Abcam (Cambridge, MA). Infrared dye-conjugated secondary antibody to rabbit was purchased from Rockland Immunochemicals (Gilbertsville, PA). siRNA to MnSOD, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a scrambled control sequence, and NeoFx Transfection Agent^{TM} were purchased from Ambion (Austin, TX). Cytotoxicity Detection Kit^{TM} was purchased from Roche Applied Science (Laval, Canada). ERbeta null and control mice were purchased from Taconic Farms (Germantown, NY). All other chemicals and purified enzymes were obtained either from Sigma–Aldrich (St. Louis, MO), BioShop (Burlington, Canada) or Fisher Scientific (Mississauga, Canada) unless otherwise stated.

5.2.2 Cell Culture

The C2C12 mouse myoblast (Sigma) cell line was cultured in accordance with the distributor's protocol. C2C12 myoblasts were cultured under conditions of 37°C, 5%

CO_2, 18% O_2 and were subcultured as required. Cell density was determined by cell counting using trypan blue exclusion to indicate viability. ERbeta null and control myoblast cell lines were generated as in Chapter 3 (Robb et al., 2012). Primary myoblasts were cultured at 37ºC, 3%O_2 and 5%CO_2.

5.2.3 Phytoestrogen Treatments

Phytoestrogens were added to serum containing culture media which includes proteins able to bind polyphenols and thus reduce their free concentrations. Each phytoestrogen was tested at 1, 10, 20 or 25, and 50μM to evaluate possible toxicity and to determine the concentration required for maximal effects on population doubling time. All phytoestrogens with the exception of glycitin, were dissolved in DMSO. Glycitin was dissolved in 95% ethanol. Media was refreshed daily and all treatments were 48h in length. Comparisons were made to the appropriate vehicle control for each compound and the data presented for each compound correspond to the concentration of that compound that yielded maximal effects on population doubling time. Where the estrogen antagonist ICI182780 was used, it was added directly to the culture media at a final concentration of 10μM 24h prior to, and throughout phytoestrogen treatment.

5.2.4 siRNA Treatment

MnSOD knockdown experiments were performed as outlined in Chapter 2.

5.2.5 Stress Resistance and Death Experiments

Stress resistance in myoblasts was measured as described in Chapter 2.

5.2.6 Lactate Dehydrogenase Activity

LDH activity was measured as described in Chapter 2.

5.2.7 Preparation of Whole Cell Lysates

Whole cell lysates were prepared following the protocol outlined in Chapter 2.

5.2.8 Western Blots

Western blots were completed following the protocol outlined in Chapter 2. 15µg of lysate protein were used for all samples. Membranes were incubated overnight at 4°C with an antibody to MnSOD (1:5000 dilution), CuZnSOD (1:1000 dilution), hsp70 (1:1000 dilution) or hsp60 (1:2000 dilution). The membranes were visualized using the Odyssey infrared imaging system from LI-COR Biosciences, with IR-linked secondary antibodies to rabbit or mouse (1:5000 dilution). Western blot analysis was performed using Odyssey imaging software 1.0.

5.2.9 Citrate Synthase Activity

Citrate synthase (CS) activity was measured as described in Chapter 2.

5.2.10 Antioxidant Enzyme Activities

Catalase and Glutathione Peroxidase activity was measured as outlined in Chapter 2.

5.2.11 Statistical Analysis:

Data for three or more experimental groups were analyzed by ANOVA. Post-hoc comparisons between means were done by Tukey's test using Systat v.12. Data comparing two experimental groups were analyzed using the student's t-test. All data are presented as means ± standard error of the mean (SEM). A p-value of < 0.05 was considered significant.

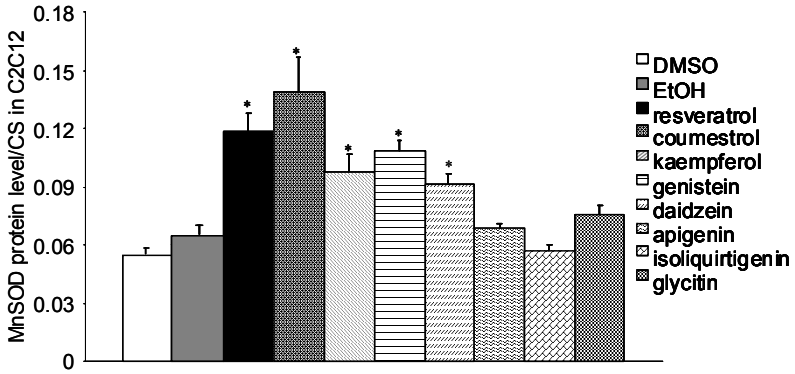
5.3 Results:

Phytoestrogen effects were evaluated in C2C12 skeletal muscle myoblasts, a cell type known to express ERs (Milanesi et al., 2008) and in which the effects of RES have been previously studied (*e.g.* Robb and Stuart 2011). The cellular effects of eight phytoestrogens (RES, coumestrol, kaempferol, genistein, daidzein, apigenin, isoliquirtigenin and glycitin) were examined at concentrations of 1, 10, 20, 25, and 50μM over a 48h period of continuous exposure (data not shown), during which culture media and phytoestrogens were refreshed after 24h. Of these concentrations, the lowest concentration capable of eliciting a maximal effect was used to evaluate effects on MnSOD expression, cell growth, and stress resistance.

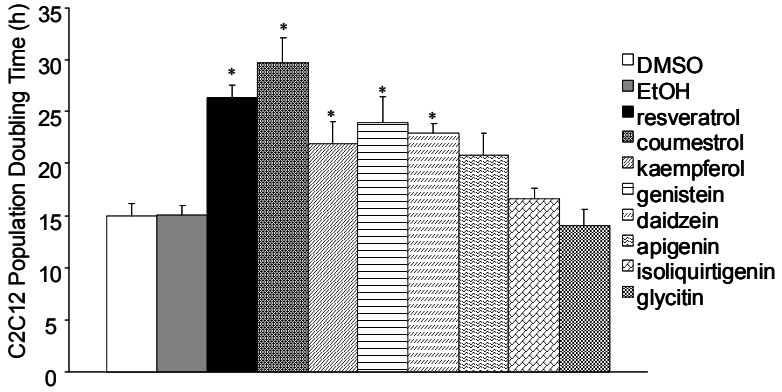
48h treatment with RES (25μM), coumestrol (10μM), kaemperfol (25μM) genistein (50μM), or daidzein (50μM) significantly increased MnSOD protein levels, while apigenin (50μM), glycitin (50μM), or isoliquirtigenin (50μM) had no significant effect on MnSOD expression (Fig. 5.2A). Estrogens and phytoestrogens including RES can stimulate a general increase in mitochondrial abundance in many cell types (e.g. Robb et al 2008a). Therefore, in these experiments, MnSOD expression was normalized to the activity of citrate synthase activity, an enzyme that correlates well with mitochondrial abundance and is frequently used as a proxy.

Consistent with the observed MnSOD induction, RES (25μM), coumestrol (10μM), kaemperfol (25μM), genistein (50μM), and daidzein (50μM) significantly slowed proliferative cell growth, shown as an increase in population doubling time (Fig. 2B). Apigenin (50μM) appeared to marginally increase population doubling time, but this change did not reach statistical significance.

5.2 A


MnSOD protein level/CS in C2C12
0.18
0.15
0.12
0.09
0.06
0.03
0
DMSO
EtOH
resveratrol
coumestrol
kaempferol
genistein
daidzein
apigenin
isoliquirtigenin
glycitin

5.2 B


C2C12 Population Doubling Time (h)
35
30
25
20
15
10
5
0
DMSO
EtOH
resveratrol
coumestrol
kaempferol
genistein
daidzein
apigenin
isoliquirtigenin
glycitin

5.2 C

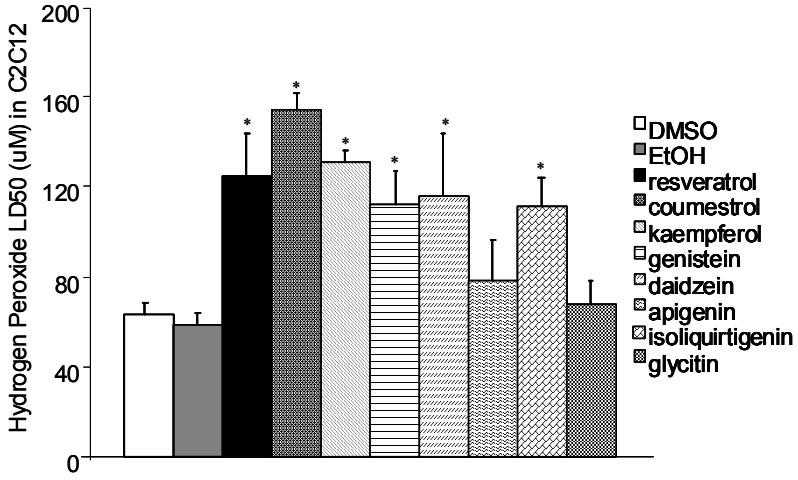


Figure 5.2. Phytoestrogens affect MnSOD protein levels, proliferative cell growth and stress resistance.
C2C12 myoblasts were treated with DMSO, ethanol, RES (25μM), coumestrol (10μM), kaemperfol (25μM), genistein (50μM), daidzein (50μM), apigenin (50μM), isoliquirtigenin (50μM) or glycitin (50μM) continuously for 48h. A. MnSOD protein levels normalized to citrate synthase activity. Data shown represent the means of 3 independent trials. B. Average population doubling time. Data shown represent the means of 4 independent trials. C. Hydrogen Peroxide LD_{50}. Data shown represent the means of 3 independent trials. Error bars represent SEM. *=$p<0.05$ compared to vehicle control.

Neither glycitin nor isoliquirtigenin had a significant effect on myoblast population doubling time at concentrations up to 50μM.

The phytoestrogens were screened for their ability to protect C2C12 myoblasts against cell death induced by the oxidant hydrogen peroxide. Since many of these compounds are themselves antioxidants capable of neutralizing exogenous hydrogen peroxide media was refreshed with new, phytoestrogen-free media 1h prior to hydrogen peroxide addition. 48h pretreatment of C2C12 cells with RES (25μM), coumestrol (10μM), kaemperfol (25μM) genistein (50μM), daidzein (50μM), or isoliquirtigenin (50μM) significantly increased myoblast stress resistance, evaluated by a change in LD50

values for hydrogen peroxide compared to vehicle control (Fig 5.2C). In contrast, neither apigenin nor glycitin had a significant effect at any concentration tested (up to 50μM).

Cellular stress resistance can be conferred by a number of mechanisms including antioxidant enzymes other than MnSOD. To determine if the phytoestrogens caused a general induction of antioxidant enzyme levels and activities, catalase and glutathione peroxidase activities, and CuZn superoxide dismutase protein levels were measured in all vehicle control and phytoestrogen treated cells. No significant differences in catalase or glutathione peroxidase activities were found following treatment with any of the eight phytoestrogens (Table 5.1). Similarly, no statistically significant change in CuZn superoxide dismutase protein levels was observed in any of the experimental groups (Table 5.1).

Table 5. 1 Effects of Resveratrol Analogues on Antioxidant Enzymes and Heat Shock Proteins. Catalase activity, Glutathione Peroxidase activity and CuZnSOD, Hsp60 and Hsp 70 protein level in C2C12 myoblasts.

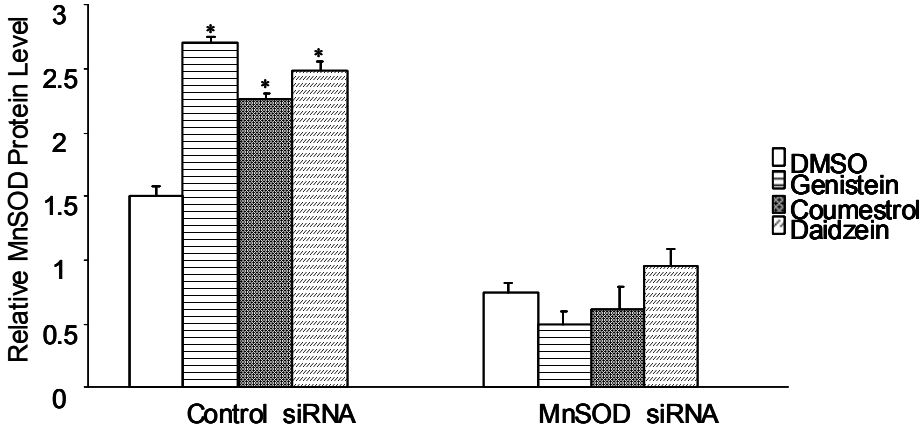
Treatment	Catalase Activity (mmol/min /mg cellular protein)	Gluathione peroxidase Activity (mmol/min /mg cellular protein	CuZn superoxide dismutase Protein Level Relative to Internal Control	Hsp60 Protein Level/Cs Activity	Hsp70 Protein Level Relative to Internal Control
DMSO	15.51±0.24	67.32±5.27	0.93±0.09	0.085±0.009	1.02±0.11
EtOH	15.80±0.47	66.52±8.14	1.10±0.12	0.087±0.012	1.07±0.08
RES	15.42±0.07	51.41±5.04	1.18±0.35	0.100±0.010	1.09±0.17
Coumestrol	16.23±0.72	54.56±7.99	0.97±0.13	0.128±0.018	1.16±0.09
Kaempferol	16.75±1.03	56.43±3.52	0.87±0.21	1.180±0.022	2.19±0.11∗
Genistein	18.13±2.07	59.38±7.48	1.08±0.24	0.094±0.016	1.54±0.23
Daidzein	16.42±0.44	53.33±2.67	1.16±0.13	0.088±0.007	1.38±0.11
Apigenin	N.D.	50.24±3.85	0.90±0.27	N.D.	1.08±0.08
Isoliquirtigenin	N.D.	46.22±8.10	0.94±0.08	N.D.	0.85±0.13
Glycitin	17.47±0.13	57.27±7.22	0.95±0.17	0.063±0.016	1.11±0.25

Data represents the mean of duplicate measurements for 3 independent trials. ∗= $p<0.05$ compared to vehicle control. N.D.= not determined

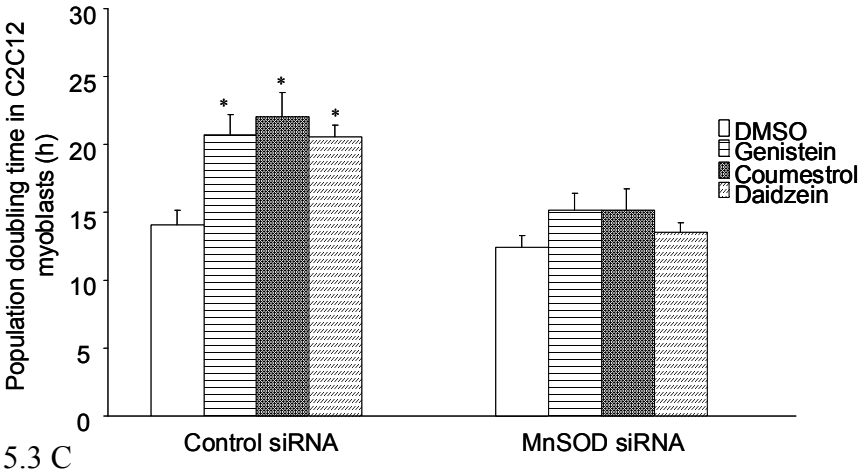
Heat shock proteins are capable of imparting stress resistance via their protein homeostasis activities (reviewed in Bukau et al., 2006). Since a phytoestrogen-stimulated upregulation of heat shock protein expression prior to stress exposure could partially explain the observation of increased hydrogen peroxide resistance associated with phytoestrogen pretreatment, the levels of Hsp70, and the mitochondrial Hsp60 were measured in treated and control cells. As with the MnSOD values, Hsp60 values were standardized to citrate synthase activity since Hsp60 is almost exclusively mitochondrial. No significant changes in the levels of Hsp60 were found in any of the treatment groups (Table 5.1). Similarly, no significant effect on Hsp70 levels was observed for any of the compounds, with the exception of kaempferol (25μM), which significantly increased Hsp70 protein levels (Table 5.1).

The phytoestrogen-stimulated induction of MnSOD thus appeared to be highly specific. To demonstrate that MnSOD upregulation was a central event in producing the phenotype of slow growth and stress resistance, siRNA was used to prevent the increase in MnSOD expression induced by phytoestrogen treatment (Fig. 5.3A). Interestingly, when the induction of MnSOD was prevented, genistein (50μM), coumestrol (10μM) and daidzein (50μM) had no significant effect on myoblast population doubling time (Fig. 5.3B). Thus, the increase in population doubling time elicited by these phytoestrogens was dependent upon their ability to upregulate MnSOD levels, and when this was prevented effects on growth were abolished (Fig. 5.3C). Similarly, under these conditions neither genistein (50μM), nor daidzein (50μM) were able to increase myoblast resistance to hydrogen peroxide (Fig. 5.3D). However, in the absence of MnSOD induction the effects of coumestrol (10μM) on myoblast stress resistance persisted.

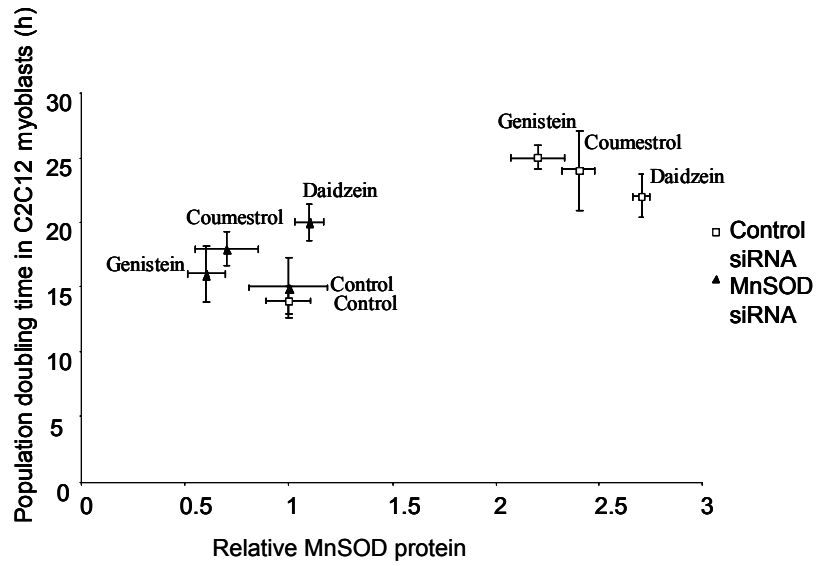
5.3 A


Relative MnSOD Protein Level
3
2.5
2
1.5
1
0.5
0
*
*
*
Control siRNA
MnSOD siRNA
DMSO
Genistein
Coumestrol
Daidzein

5.3 B


Population doubling time in C2C12 myoblasts (h)
30
25
20
15
10
5
0
*
*
*
Control siRNA
MnSOD siRNA
DMSO
Genistein
Coumestrol
Daidzein

5.3 C


Population doubling time in C2C12 myoblasts (h)
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25
20
15
10
5
0
0
0.5
1
1.5
2
2.5
3
Relative MnSOD protein
Daidzein
Coumestrol
Genistein
Control
Control
Genistein
Coumestrol
Daidzein
Control siRNA
MnSOD siRNA

5.3 D

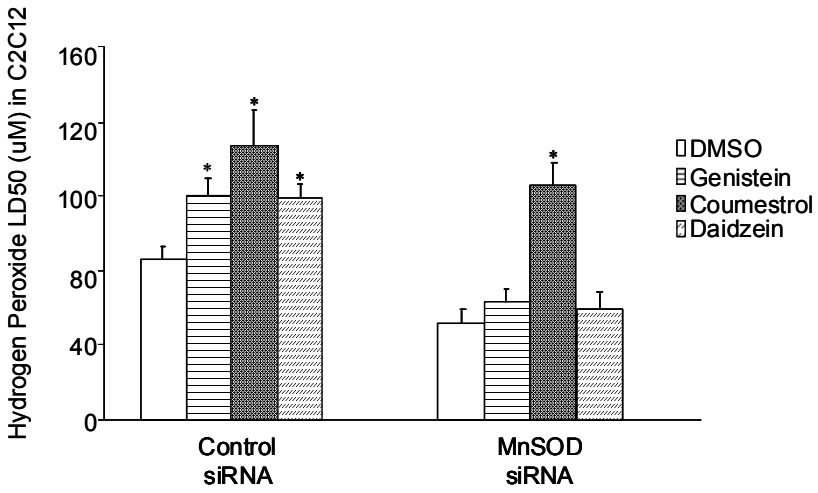


Figure 5.3. MnSOD is essential for phytoestrogens' effects on cellular stress resistance and population doubling time.
C2C12 myoblasts were treated with control siRNA or MnSOD siRNA ± genistein (50μM), coumestrol (10μM), or daidzein (50μM). A. Relative MnSOD protein level. B. Average population doubling time. C. Correlation of MnSOD protein level and population doubling time. D. Hydrogen Peroxide LD_{50}. Data shown represents the mean of 3-4 independent trials. Error bars represent SEM. *=$p<0.05$ compared to vehicle control.

To determine if the effects of the phytoestrogens that induced MnSOD were being mediated through estrogen receptors, the initial phytoestrogen treatment experiments were repeated with cells that had been pretreated for 24h with the estrogen antagonist ICI182780 (10 μM), which was maintained throughout the subsequent 48h phytoestrogen treatment. Treatment with ICI182780 eliminated the effects of RES (25μM), coumestrol (10μM), kaemperfol (25μM) genistein (50μM) and daidzein (50μM) on MnSOD protein levels, population doubling time, and resistance to hydrogen peroxide induced cell death (Table 5.2), indicating the importance of estrogen signaling to these three effects.

Table 5.1: Phytoestrogen Effects on Population Doubling Time, Resistance to Hydrogen Peroxide Induced Cell Death, and MnSOD Protein Level in the Absence and Presence of ICI182780.

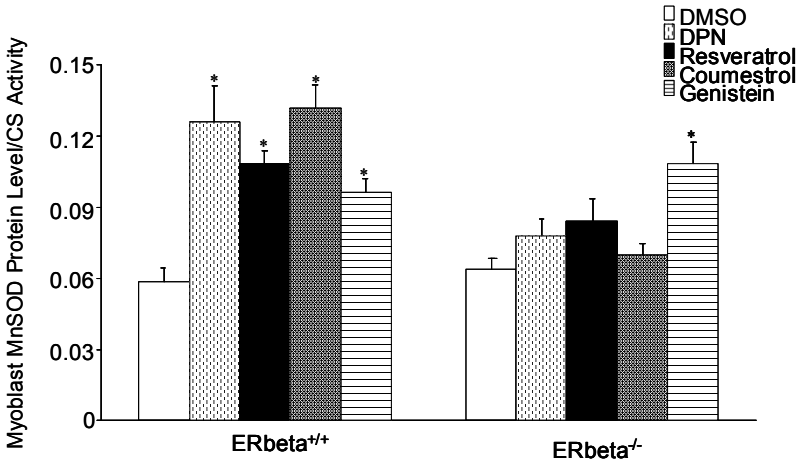
Treatment	Population Doubling Time (h)		H_2O_2 LD_{50} (μM)		MnSOD Protein Level Relative to Internal Control	
	No Antagonist	Antagonist	No Antagonist	Antagonist	No Antagonist	Antagonist
DMSO	14.98±1.16	15.29±0.11	92±11	82±07	1.06±0.15	0.94±0.12
EtOH	15.01±0.94	14.87±0.16	84±09	93±06	1.10±0.18	1.04±0.08
Resveratrol	26.34±1.25∗	17.23±1.22	144±21∗	96±10	2.10±0.14∗	0.83±0.09
Coumestrol	29.72±2.39∗	14.21±0.30	177±09∗	109±11	2.46±0.23∗	1.18±0.32
Kaempferol	21.87±2.12∗	14.51±2.01	150±10∗	59±18	1.72±0.16∗	1.02±0.14
Genistein	23.92±2.02∗	18.20±1.84	129±07∗	80±21	1.92±0.18∗	1.14±0.26
Daidzein	22.92±0.96∗	15.66±1.43	133±06∗	86±08	1.61±0.08∗	1.22±0.38
Glycitin	14.02±1.57	14.99±0.88	84±14	96±09	1.17±0.12	1.20±0.15

Antagonist = ICI182780. Data represents the mean of duplicate measurements for 3 independent trials. ∗= $p<0.05$ compared to vehicle control

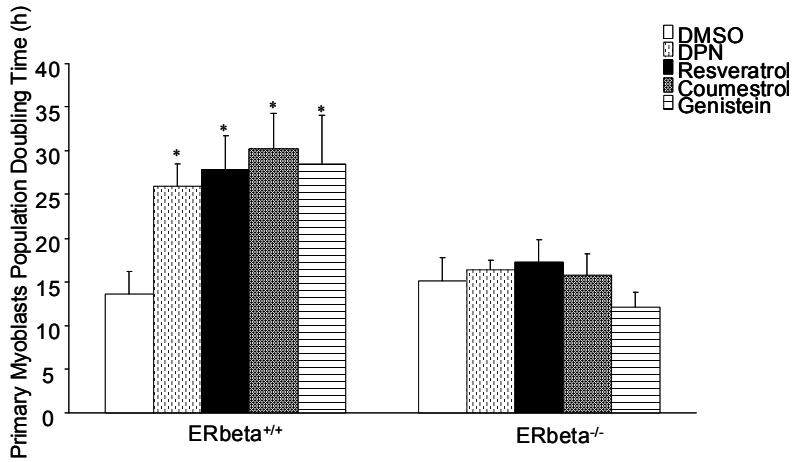
Longer-term effects of estrogen signaling are predominately mediated by two receptors: ERalpha and ERbeta. Similar to what is observed with 48h of phytoestrogen treatment, the ERbeta agonist DPN, but not the ERalpha agonist PPT, increases population doubling time and stress resistance in C2C12 myoblasts (Robb and Stuart, 2011; Chapter 2). This data supports the idea that ERbeta is an important component of the signaling pathway mediating the effects of phytoestrogens. Myoblast cell lines derived from ERbeta null mice were used to evaluate the specific role of ERbeta in mediating the effects of the phytoestrogens used in this study. In myoblasts, the absence of ERbeta abolished the effects of DPN (10 μM), RES (25μM), and coumestrol (10μM) on MnSOD expression while, surprisingly, genistein significantly increased MnSOD protein levels in the ERβ null cell lines (Fig 5.4A). Consistent with these observations, the effects of DPN, RES and coumestrol on population doubling time were absent in ERbeta null cells (Fig 5.4B). Although genistein is reported to be an

ERbeta agonist, genistein treatment appeared to actually decrease population doubling time of ERbeta null myoblasts (Fig 5.4B). While the effects on hydrogen peroxide resistance were absent in ERbeta null myoblasts treated with RES or DPN, surprisingly they persisted in ERbeta null cells treated with either coumestrol or genistein (Fig. 5.4C).

5.4 A



5.4 B



5.4 C

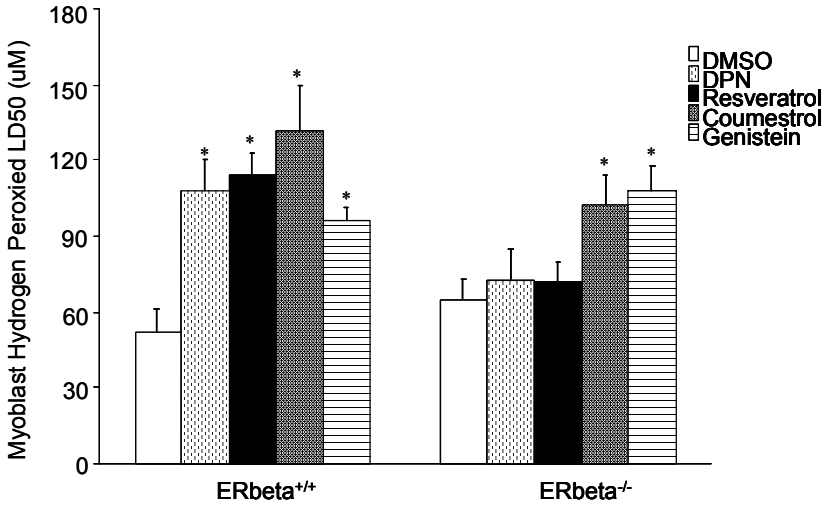


Figure 5.4. Involvement of ERbeta in phytoestrogen effects on population doubling time, stress resistance and MnSOD protein level.
Control or ERβ-null myoblasts treated with DMSO, DPN (10μM), RES (20μM), coumestrol (10μM) or genistein (50μM). A. MnSOD protein levels normalized to citrate synthase activity in control and ERbeta null myoblasts. B. Average population doubling time. C. Hydrogen Peroxide LD_{50}. Data shown represents the mean of 5 independent trials. Error bars represent SEM. *=$p<0.05$ compared to vehicle control.

5.4 Discussion:

Of the seven phytoestrogens investigated here, coumestrol, kaempferol, genistein, and daidzein were capable of eliciting a similar induction of MnSOD that had been previously observed with RES and the ERbeta-specific agonist DPN (Robb and Stuart 2011; Chapter 2). Although the cellular effects of phytoestrogens have been suggested to involve their ability to modulate antioxidant enzyme activity via interactions with redox sensitive transcription factors (Siow and Mann 2010; Froyen and Steinberg 2011), there was no evidence for a broad induction of antioxidant enzymes in the experiments outlined here. None of catalase, glutathione peroxidase or superoxide dismutase was significantly increased following phytoestrogen or DPN treatment. Notably, coumestrol, kaempferol, genistein, and daidzein all slowed cell growth and enhanced resistance to hydrogen peroxide, effects associated with MnSOD overexpression. The induction of MnSOD expression appeared to be critical for the growth inhibitory effects of genistein, coumestrol and daidzein, since it was abolished when siRNA was used to prevent it. Similarly, with the exception of coumestrol, enhanced resistance to hydrogen peroxide toxicity also required the induction of MnSOD. Thus coumestrol, genistein, daidzein, and perhaps other phytoestrogens are all specific inducers of MnSOD expression, producing a phenotype (slowed growth and cytoprotection) essentially the same as that achieved with transgenic overexpression of MnSOD. It is possible that many phytoestrogens other than those investigated here are specific stimulators of MnSOD expression and achieve their growth inhibitory and cytoprotective activities by this central mechanism.

Some of the published observations of phytoestrogens inhibiting proliferative growth and conferring cytoprotection might therefore be explained by the ability of these compounds to upregulate MnSOD expression. In addition to previous observations made with RES (Robb and Stuart 2011; Chapter 2), an increase in MnSOD has also been previously reported in response to genistein treatment (Borrás et al., 2006; Xi et al., 2011). MnSOD has also been previously identified as a downstream target of estrogen. Estrogen treatment increases MnSOD protein levels in rat aortic vascular smooth muscle cells, a cancerous mammary cell line (MCF-7) and mouse myoblasts (C2C12) (Larosche et al., 2010; Sivritas et al., 2011; Borrás et al., 2005; Robb and Stuart, 2011). Consistent with this, females generally have higher brain levels of MnSOD than males (Viña et al., 2006). Thus, estrogens, RES and genistein are all known to induce MnSOD expression in a wide variety of cell types, both *in vivo* and *in vitro*. However, there has been no data concerning the effects of other phytoestrogens on MnSOD expression. This data is the first to show that MnSOD is a downstream target of multiple phytoestrogens and that the upregulation of its expression underlies effects on cell growth and stress resistance that are of interest in the context of phytoestrogen supplementation.

Estrogen signaling is mediated mainly by ERalpha and ERbeta, and the C2C12 cell line used here express both ER forms (Milanesi et al., 2008). Similar to what is observed with MnSOD overexpression alone, (Weydert et al., 2003; Ough et al., 2004) ERbeta reduces proliferative cell growth under a variety of experimental conditions. Treatment of the colon cancer cell line MC38 with the specific ERbeta agonist DPN significantly reduces cell proliferation (Motylewska et al., 2008). *In vivo* DPN administered to Apc(Min/+) mice significantly reduces their incidence of intestinal

tumours compared to untreated controls (Giroux et al., 2011). Lentivirus transfection of ERbeta into a cancerous colon cell line significantly reduces cell proliferation, even without the addition of ERbeta specific agonists (Hartmanet al., 2009). The effects of ERbeta on MnSOD in the experiments outlined above are unknown, and this report is the first to make the connection between ERbeta binding and MnSOD.

Many of the phytoestrogens used in this study have been reported to bind preferentially to ERbeta. Unlike RES, genistein continued to slow replicative cell growth, enhance stress resistance and increase MnSOD protein level in the absence of ERbeta. Thus, the requirement for ERbeta did not apply to all of the phytoestrogens tested. This is not surprising given that there is considerable overlap in the structures of ERalpha and ERbeta that may permit phytoestrogens to bind to both forms with an affinity for one receptor over the other that is dependent on specific aspects of the phytoestrogen's structure. A recent computational study by Yuan et al. (2011) reported that genistein did not show a strong binding preference for either ERbeta over ERalpha, while RES showed a strong affinity for ERbeta. These structure specific affinities are likely to play an important role in predicting the effects of phytoestrogens in tissues and cell types with different ERalpha ERbeta ratios.

One criticism that has been made of *in vitro* experiments with phytoestrogens is that the concentrations required to achieve the desired effects are higher than can be achieved by dietary supplementation in vivo. Two observations suggest that the effects seen *in vitro* can be generally translated to *in vivo*. Firstly, the experiments presented here were done in media containing 10% fetal bovine serum (FBS), which contains proteins that effectively bind the generally hydrophobic phytoestrogens. For example,

within 8h of addition of RES to media containing 10% FCS less than 10% of RES remains free; by 24h only a few percent remains unbound (Colin et al., 2011). Thus, although these compounds might be added to media at 10-50 μM it is expected that the effective free concentration is much lower. Secondly, although dietary supplementation with a variety of phytoestrogens results in generally rather low plasma bioavailability (*e.g.* Yuan et al., 2011) the expected results are often observed nonetheless. For example, dietary RES administration elevates brain (Robb et al., 2008b; Chapter 6) and skeletal muscle (Jackson et al., 2010; Jackson et al., 2011) MnSOD levels, and also confers neuroprotection (Shan et al 2007; Dumont et al 2009) and oxidative stress resistance, respectively. Similarly, dietary supplementation with RES slows the growth of a variety of cancers *in vivo* despite its low bioavailability (reviewed in Scott et al, 2012). Thus dietary delivery can achieve similar effects in vivo as have been characterized in vitro, suggesting that in vitro models for determining mechanisms of action provide real insight.

The phytoestrogens investigated in this study elicit similar cellular effects and appear to share a common mechanism; therefore the challenge of a single compound's limited bioavailability may be circumvented by combining many phytoestrogens to reach the concentrations necessary for their effects in vivo. While potential synergisms between these compounds were not studied here here, Kumar et al. (2011) recently reported that a combination of genistein, quercetin and biochanin A was more effective at inhibiting the growth of prostate cancer cell lines than a single dose of either compound (Kumar et al., 2011). Further research is required to determine if there are any synergetic

interactions between the phytoestrogens that could be used to enhance their in vivo activities.

In conclusion, the mitochondrial antioxidant enzyme MnSOD plays an important role in the anti-proliferative and cytoprotective properties of several common dietary phytoestrogens. Further, these effects are mediated by ERs, since they are abolished by the ER antagonist ICI182780. Finally, ERbeta in particular is, at least for some phytoestrogens, is involved in mediating the induction of MnSOD and resultant phenotypic effects. This mechanistic information will be useful in realizing the full nutraceutical potential of phytoestrogens.

Chapter 6: Dietary Resveratrol Administration Increases MnSOD Protein Levels and Activity in Mouse Brain

Hypothesis: MnSOD is an important target of RES *in vitro*. I hypothesize that RES given to mice will increase the protein level and activity of MnSOD in highly metabolic tissues: brain, heart, liver and kidney.

Objectives: The objectives of this project were to examine the effects of RES on antioxidant enzymes in mice given RES in: 1) a high fat diet, 2) a standard laboratory diet and 3) via subcutaneous osmotic minipump.

Publications of results:

Robb EL, Winkelmolen L, Visanji N, Brotchie J, Stuart JA. Dietary resveratrol administration increases MnSOD expression and activity in mouse brain. Biochemical and Biophysical Research Communications. 372: 254-259, 2008.

Contributions:

I performed all experiments, statistical analysis and chapter write-up.

6.1 Introduction:

RES, a bioactive component of red wine, has become well known for its reported ability to extend lifespan in model organisms ranging from yeast to vertebrates (Howitz et al., 2003; Wood et al., 2004; Bauer et al., 2004; Valenzano et al., 2006). In addition to lifespan extension, RES has also shown putative protective actions against neurodegeneration, cancer, cardiovascular disease, diabetes and the detrimental effects associated with high fat diets (Baur and Sinclair, 2006; Baur et al., 2006). Oxidative stress is a shared observation in many of these pathologies and resistance to oxidative stress is a strong correlate of lifespan potential (Finkel and Holbrook, 2000). A RES induced decrease in cellular oxidative stress may provide a mechanism by which this polyphenol is able to exert a wide range of beneficial effects. Although RES has antioxidant properties related to the presence of its phenolic hydroxyl groups, low bioavailability and a weak ability to directly scavenge reactive oxygen species, (ROS), makes cytoprotection via direct chemical reactions unlikely (Leonard et al., 2003; Sale et al., 2004). A more plausible hypothesis is that RES initiates a cascade of intracellular events that lead to an upregulation of cellular defense systems, which in turn protect against oxidative stress.

Interactions between RES and intracellular signaling molecules including sirtuins and the fork head family of transcription factors have been reported both *in vivo* and *in vitro* (Baur et al., 2006; Stefani et al., 2007; Lagouge et al., 2006). Recent studies suggest that activation of SIRT1, and its target PGC-1α, by RES in mice leads to changes in mitochondrial number and function (Lagouge et al., 2006). As the primary source of ROS production in most cell types (Finkel and Holbrook, 2000) mitochondria are

important components of responses aimed at decreasing oxidative stress. The mitochondrial isoform of superoxide dismutase, MnSOD, is therefore a downstream target of many signaling pathways proposed to mediate cellular stress resistance (Kops et al., 2002). RES is able to induce MnSOD in a human lung fibroblast cell line (MRC-5) (Robb et al., 2008a). An elevation of MnSOD *in vivo* in response to RES would be a significant finding given the enzyme's importance in various models of disease (Macmillan-Crow and Cruthirds, 2001). Many of RES's reported *in vivo* effects are consistent with an increase in mitochondrial ROS metabolism; however, observation of antioxidant enzyme activities, including MnSOD, in normal mice following chronic RES treatment has not yet been reported. It is hypothesized that an important action of RES may be to reduce intracellular oxidative stress by increasing mitochondrial ROS metabolism.

The aim of this study was to examine antioxidant enzymes in the brain, heart and liver of mice administered RES for four consecutive weeks. To assess the influence of delivery method three routes of RES administration were tested: Incorporation into a standard laboratory diet, incorporation into a high fat diet or delivery via a subcutaneous osmotic minipump. Here, it is reported that both dietary and subcutaneous RES delivery methods are capable of altering the activities of key antioxidant enzymes glutathione peroxidase, catalase and MnSOD, and increasing mitochondrial content in heart, brain and liver.

6.2 Experimental Procedures:

6.2.1 Materials

C57 BL6 mice were obtained from Charles River Laboratories (Charles River, Canada). Alzet 2004 minipumps were purchased from Alzet (Cupertino, USA). trans-RES (purity >95%) was purchased from ChromaDex Inc (Irvine, USA). Mouse chow, AIN-93G and AIN-93G modified (60% calories from fat), was purchased from DYETS (Bethlehem, USA). Chemicals were purchased from Sigma-Aldrich (Oakville, Canada) and BioShop (Burlington, Canada). BioRad protein dye was obtained from BioRad laboratories, (Hercules, USA). Prestained broad range protein marker was obtained from New England BioLabs, (Ipswich, USA). MemCode reversible protein stain kit was purchased from Pierce Biotechnology, (Rockford, USA). Antibodies to human CuZnSOD and human MnSOD were purchased from StressGen Biotechnologies (Ann Arbor, USA). IR dye-conjugated secondary antibodies to rabbit and mouse were purchased from Rockland Immunochemicals, (Glibertsville, USA).

6.2.2 Animal Care Conditions

Male C57 BL6 mice (Charles River, Canada) were housed in groups of three in a temperature and humidity controlled environment subject to a 12 hour light/dark cycle. Standard mouse chow and water were available ad libitum to the minipump treatment groups. Dietary delivery groups were given controlled access to food, water was available ad libitum. All treatment protocols adhered to the institutions animal care guidelines.

6.2.3 Resveratrol Treatment

At 5 weeks of age, mice were introduced to RES by one of three delivery methods; in a high fat diet, a standard laboratory diet or through a subcutaneous osmotic minipump (MP). RES was incorporated into mouse chow of both standard and high fat diets at a concentration of 0.1% (w/w), such that mice obtained a RES dose of approximately 200mg/kg/day. Alzet 2004 minipumps were preloaded with 50% degassed DMSO or 1.83M RES prepared in 50% degassed DMSO. The minipumps were implanted subcutaneously under isoflorane anesthesia and released vehicle or RES at a flow rate of 0.25µL/hour to give a dosage of 100mg/kg/day. The standard diet (SD) group contained 8 control mice and 8 RES-treated mice. The high fat diet (HFD) group contained 6 control mice and 8 RES-treated mice. The osmotic minipump group contained 5 control mice and 8 RES-treated mice.

6.2.4 Tissue Harvesting:

Animals were sacrificed by cervical dislocation at the end of 4 weeks of treatment. Brain, liver and heart were removed and immediately frozen on dry ice, then transferred to -80°C for preservation.

6.2.5 Tissue Homogenization:

Frozen tissues were homogenized in 2 volumes of ice cold buffer containing 10mM KH_2PO_4 (pH 7.3), 30mM KCl, 20µM EDTA and 0.1% Triton X-100 using a polytron homogenizer. The homogenates were centrifuged at 500g for 10 minutes. The resulting supernatant was collected and protein concentration was determined by the Bradford method using the BioRad™ Assay.

6.2.6 Enzyme Activities:

Citrate synthase activity was assayed as outlined in Chapter 2.

Catalase activity was assayed as outlined in Chapter 2.

Glutathione Peroxidase activity was assayed as outlined in Chapter 2.

The activities of MnSOD and CuZn superoxide dismutase were measured using an in-gel assay as has been previously described (Beauchamp and Fridovich, 1971; Robb et al., 2008a). Briefly, native gel electrophoresis was used to separate and quantify the activity of SOD isoforms. Following electrophoresis, gels were incubated in a solution of 1.23 mM nitro blue tetrazolium (r.t.). Gels were rinsed with water and stained with a solution of 28mM TEMED and 2.8 x 10^{-2}mM riboflavin in potassium phosphate buffer, pH 7.8. To distinguish between CuZn superoxide dismutase and MnSOD bands, 5mM KCN was added to the staining solution in some gels. Gels were then washed with water and illuminated under bright fluorescent light until the background had developed to a uniform blue-violet colour and bands were clearly visible. Quantification was achieved using an in-gel standard curve constructed from a dilution series of pure bovine liver SOD. Gels were scanned and SOD activities were quantified using Bio-Rad's Quantity One® software.

6.2.7 Western Blotting

Western blots were performed as outlined in Chapter 2, using equal amounts of crude homogenate protein (20μg). The arbitrary luminescence values of MnSOD bands in each Western were normalized to an internal standard.

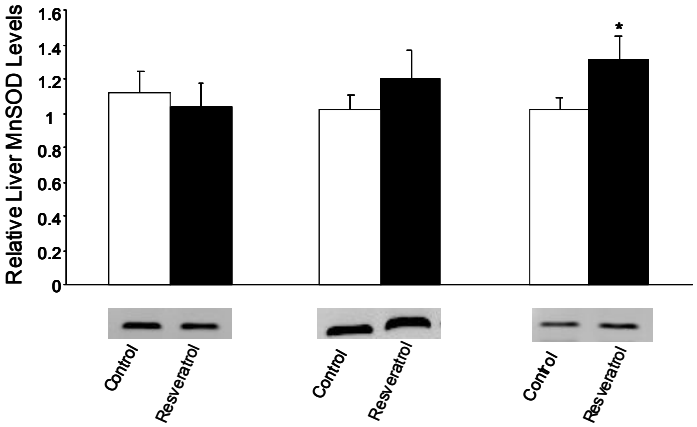
6.2.8 Statistical Analysis

Data collected were analyzed by ANOVA, followed by Tukey's post hoc test. Statistical analysis was performed using Systat. $p<0.05$ was taken as significant. Error bars represent SEM.

6.3 Results:

The effects of RES on the cytosolic and mitochondrial isoforms of superoxide dismutase were determined by Western blotting to detect CuZn superoxide dismutase and MnSOD. CuZn superoxide dismutase protein levels were unchanged by RES in all tissues and experimental groups (data not shown). In contrast, the protein level of the mitochondrial isoform, MnSOD, was found to vary in response to RES administration and delivery method. Administration of RES in a standard diet was ineffective in modulating MnSOD protein levels in all tissues examined. In liver tissue of the minipump delivery group, MnSOD was elevated with RES treatment when compared to the DMSO vehicle control (Fig 6.1A). A substantial increase in MnSOD protein level in brain was observed when RES was given in a high fat diet. Interestingly, administration of RES through an osmotic minipump, which was hypothesized to provide the highest circulating levels of RES, was ineffective in raising MnSOD protein levels above control values in brain (Fig 6.1B). In contrast to observations made in brain tissue, MnSOD levels decreased in heart tissue of mice fed RES in a high fat diet (Fig 6.1C). To ascertain whether changes in protein level were reflective of enzyme activity, SOD activity was measured. Changes in MnSOD protein levels in the high fat treatment method corresponded to a parallel increase, or decrease, in enzyme activity (Fig 6.1D).

6.1 A


Relative Liver MnSOD Levels
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1.4
1.2
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0.8
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0.4
0.2
0
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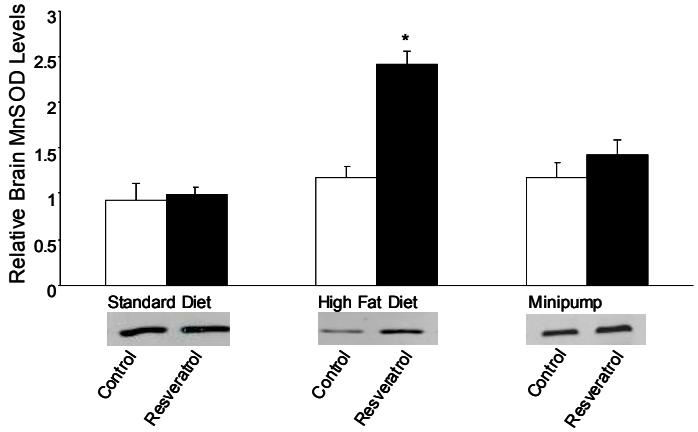
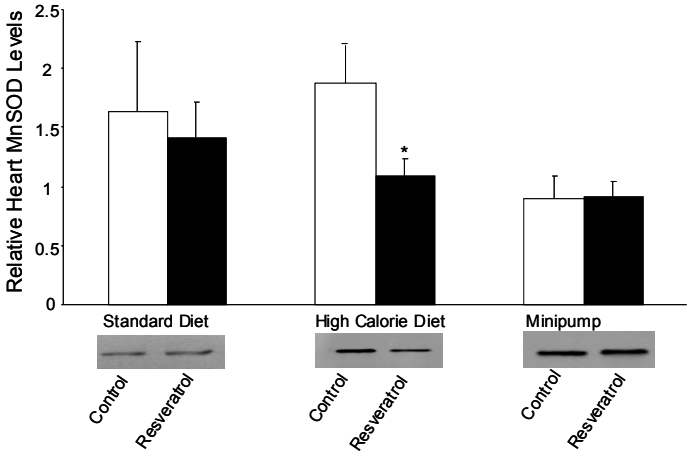
Control
Resveratrol
Control
Resveratrol

6.1 B


Relative Brain MnSOD Levels
3
2.5
2
1.5
1
0.5
0
*
Standard Diet
High Fat Diet
Minipump
Control
Resveratrol
Control
Resveratrol
Control
Resveratrol

6.1 C


Relative Heart MnSOD Levels
2.5
2
1.5
1
0.5
0
*
Standard Diet
High Calorie Diet
Minipump
Control
Resveratrol
Control
Resveratrol
Control
Resveratrol

6.1 D

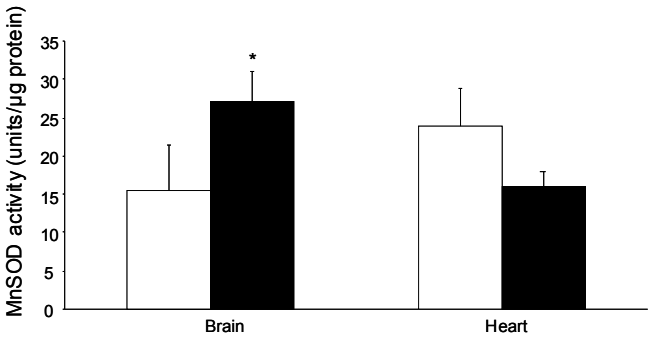


Figure 6.1. MnSOD protein level and activity in brain, heart and liver tissue of control (open bars) and RES (solid bars) groups of three treatment methods. Relative changes in MnSOD protein level in tissue homogenates and representative western blots showing MnSOD protein band in A. liver homogenates B. brain homogenates and C. heart homogenates. Relative change was measured using an internal standard as a reference and values were interpolated form a standard curve. Values shown are means ± SEM of duplicate western blots for each homogenate. D. Activity of MnSOD in brain and heart of high fat diet method. Values shown are means ± SEM of duplicate measurements of 4 homogenates per group. (SD, Con n=8, Res n=8; HFD Con n=6, Res n=8; MP Con n=5, Res=7). * = significantly different from control group ($P<0.05$).

Administration of RES in a high calorie diet has been reported to increase mitochondrial number in liver, skeletal muscle and brown adipose tissue (Baur et al., 2006; Lagouge et al., 2006). Therefore, to determine if any observed changes in MnSOD, a mitochondria specific antioxidant, were a result of a change in the number of mitochondria, citrate synthase (CS) activity was measured. Citrate synthase is a citric acid cycle enzyme whose activity is commonly measured as a proxy of mitochondrial number. Citrate synthase activity was unchanged in liver of RES treated mice in all three delivery methods (Fig 6.2A). Citrate synthase activity was increased in brain tissue of mice given RES in high fat diet and through an osmotic minipump (Fig 6.2B). An increase in citrate synthase activity was also observed in heart tissue of mice given RES in high fat diet, while a decrease in mitochondrial number was observed in the

minipump treatment group (Fig 6.2C). MnSOD protein levels were normalized to citrate synthase activity to account for any changes in protein level due to increased mitochondrial number. With mitochondrial number accounted for, changes in MnSOD protein level continued to follow the same trends (data not shown).

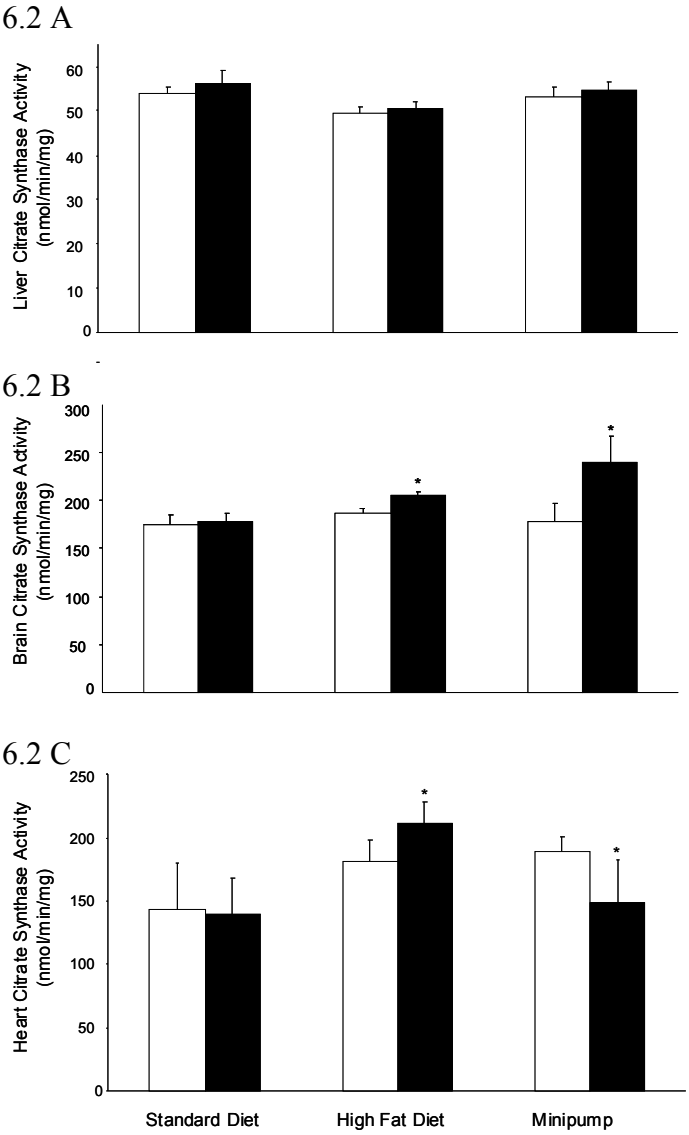
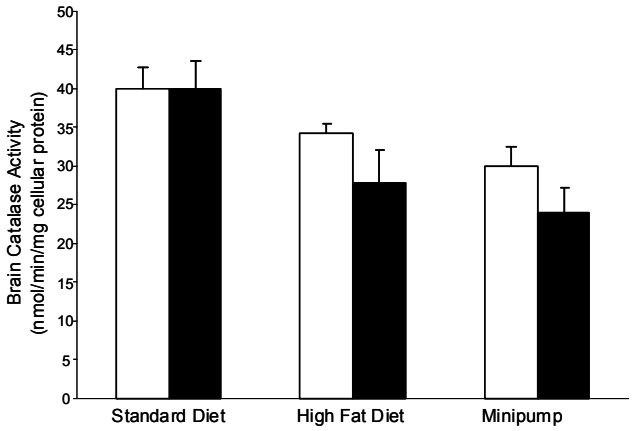


Figure 6.2. Citrate synthase activity in brain, heart and liver tissue of control (open bars) and RES (solid bars) groups of three treatment methods.
A. Citrate synthase activity in liver homogenates B. Citrate synthase activity in brain homogenates and C. Citrate synthase activity in heart homogenates. Values shown are means ± SEM of duplicate measurements of each homogenate. SD, Con n=8, Res n=8; HFD Con n=6, Res n=8; MP Con n=5, Res=7.
* = significantly different from control group ($P<0.05$)

The effect of RES on cellular antioxidant enzyme capacity was further analyzed by measuring the activities of catalase and glutathione peroxidase, two enzymes that participate in the removal of hydrogen peroxide. RES delivery though a standard laboratory diet failed to change the activity of catalase in any of the examined tissues. An induction of catalase activity in heart tissue of mice administered RES through a high fat diet or osmotic minipump was observed (Fig. 6.3B), while this effect was absent in liver (data not shown) and brain tissue (Fig. 6.3A).

6.3 A



6.3 B

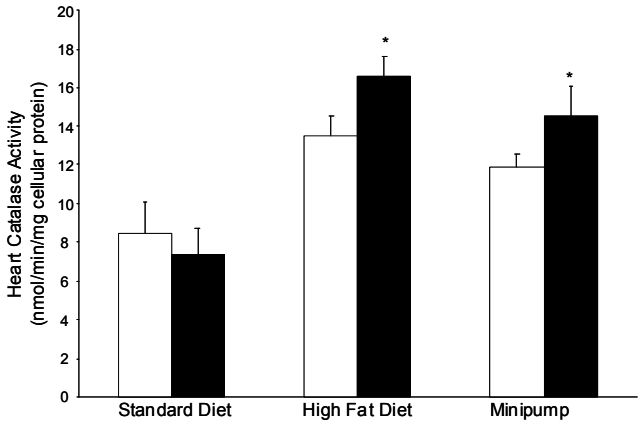
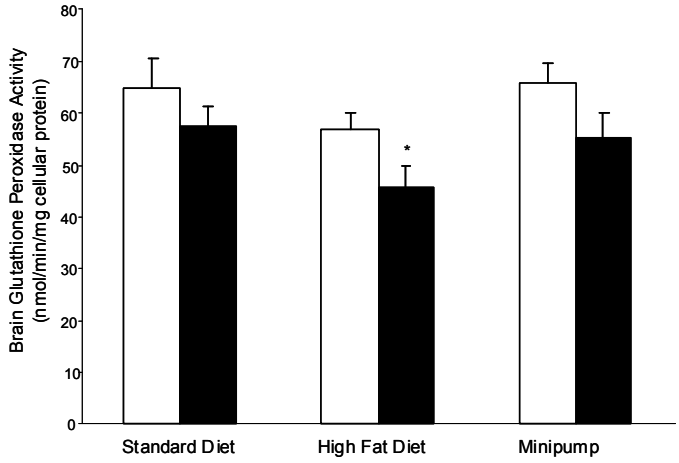


Figure 6.3. Catalase activity in brain and heart tissue of control (open bars) and RES (solid bars) groups of three treatment.
A. Catalase activity in brain homogenates B. Catalse activity in heart homogenates. Values shown are means ± SEM of duplicate measurements of each homogenate. SD, Con n=8, Res n=8; HFD Con n=6, Res n=8; MP Con n=5, Res=6. * = significantly different from control group ($P<0.05$)

A subtle decrease in glutathione peroxidase activity was observed in brain tissue of mice treated with RES through high fat diet or osmotic minipump (Fig 6.4A). In contrast, glutathione peroxidase activity was increased in heart cells of mice given RES through a high fat diet. A trend toward increasing glutathione peroxidase activity in the heart tissue of the RES minipump treatment group was also observed, although the effect did not reach statistical significance (Fig 6.4B).

6.4 A



6.4 B

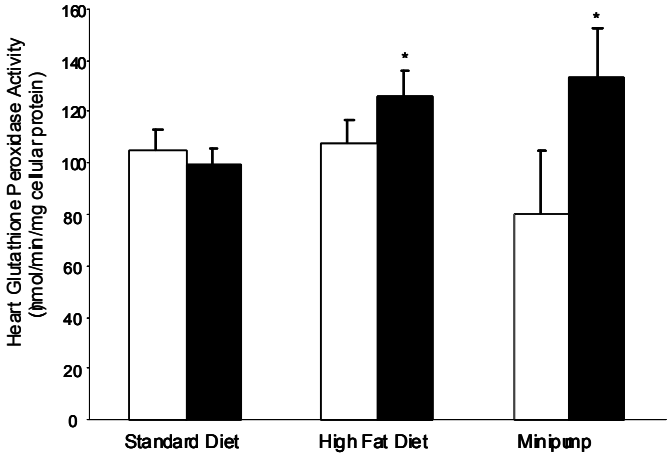


Figure 6.4. Gluathione peroxidase activity in brain and heart tissue of control (open bars) and RES (solid bars) groups of three treatment methods.
A. Glutathione peroxidase activity in brain homogenates B. heart homogenates. Values shown are means ± SEM of duplicate measurements of each homogenate. SD, Con n=8, Res n=8; HFD Con n=6, Res n=8; M.P. Con n=5, Res=7. * = significantly different from control group ($P<0.05$)

6.4 Discussion:

Recently, RES has been reported to improve the mitochondrial function of mice on a high calorie diet (Baur et al., 2006; Lagouge et al., 2006). An improved ability to metabolize mitochondrial ROS may play an important role in this observation, based on the previous finding that chronic RES treatment dramatically upregulates MnSOD in human cells *in vitro* (Robb et al., 2008a). MnSOD is the only superoxide dismutase present in the mitochondrial matrix, and is capable of reducing intracellular oxidative stress. Overexpression of MnSOD increases resistance to mitochondrial dysfunction, permeability transition and apoptotic death invoked by oxidative stress in various disease contexts (Silva et al., 2005; Kanwar et al., 2007; Kowluru et al., 2006; Klivenyi et al., 1998; Callio et al., 2005).

It is therefore interesting that RES administered in a high fat diet induced a significant increase in MnSOD protein level (140%) and activity (75%) in brain tissue that could not be explained simply by proliferation of mitochondria (10%). This is an important observation given that MnSOD overexpression alone is neuroprotective, ameliorating oxidative damage in response to ischemic events and chemical stressors such as MPTP (Klivenyi et al., 1998; Callio et al. 2005; Keller et al., 1998; Shan et al., 2007). RES has also been shown to protect against neuronal death (Bureau et al., 2008; Alvira et al., 2007; Okawara et al., 2006) and is therefore of interest for its potential ability to protect against neurodegeneration (Anekonda, 2006). MnSOD is a downstream target of RES that may play a role in the neuroprotective effects of this polyphenol. From the present study, it is clear that the mode of delivery is important in determining RES's effects. Improved delivery methods, or longer term treatments may allow for

further elevation of MnSOD expression in the brain. In any event, it appears that RES could represent a safe dietary means by which increased MnSOD expression can be achieved in brain, and neuroprotective benefit realized.

MnSOD protein level has been shown to vary between different regions of the brain (Campese et al., 2007); however, whether this reflects differences in MnSOD per mitochondrion, or simply differences in the mitochondrial content of neurons within different brain regions is unclear. While evidence of MnSOD induction with RES may support its use to prevent oxidative stress associated with neurodegenerative disease, this experiment was conducted with whole tissue homogenates, and it is therefore not possible to determine whether specific areas of the brain are influenced more than others. It will be interesting to examine specific brain regions, such as the substantia nigra, to determine whether the effects of RES are highly localized.

RES has many cardio-protective properties (Baur and Sinclair, 2006). For example, a previous study reported increased levels of SOD in cultured cardiomyocytes (H9C2 cells) treated with micromolar concentrations of RES (Li et al., 2006). Nonetheless, under the conditions used in this study a decrease in MnSOD protein level and activity was observed. RES did, however, induce subtle increases in the activities of glutathione peroxidase and catalase in heart tissue of both the high calorie and minipump groups. This agrees with previous observations made in vascular tissue treated with RES. In these experiments glutathione peroxidase and catalase were found to be critical in RES's protective effects against oxidative stressors in cultured aortic preparations (Ungvari et al., 2007). It is therefore interesting that changes in these enzymes were also observed *in vivo*. The net result of reduced MnSOD activity

concomitant with increased capacity to remove H_2O_2 should be a reduction in H_2O_2 concentrations, which could also be protective in cardiomyocytes.

In addition to altering MnSOD levels, RES has been reported to interact directly with mitochondrial oxidative phosphorylation (Zini et al., 1999; Zheng and Ramirez, 2000; Gledhill et al., 2007) and biogenesis (Baur et al., 2006; Lagouge et al., 2006; Dasgupta, and Milbrandt, 2007). One observation that has been made is that RES treatment in a high fat diet increases mitochondrial abundance in liver, brown adipose tissue and skeletal muscle of mice (Baur et al., 2006; Lagouge et al., 2006). Interestingly, no increase in citrate synthase activity (a proxy of mitochondrial number) was observed in liver, despite using a comparable dose of RES in the same strain of mice. This may be due to differences in the length of treatment time and the age of the mice at the onset of RES treatment. However, significant increases in citrate synthase activity were observed in brain and heart tissue of the high fat RES group. Therefore, it seems that oral administration of RES is capable of inducing mitochondrial proliferation in a variety of highly oxidative tissues. The mechanism and significance of this potentially important observation remain to be determined. It may represent a general shift toward a more oxidative metabolism, as is observed in caloric restriction (Baur et al., 2006). However, genetic manipulations that increase MnSOD have also been shown to increase mitochondrial oxidative capacity (Silva et al., 2005). Therefore, the improved mitochondrial and physiological function observed in rodents treated with RES administered in high fat diets may be directly linked to the induction MnSOD or other antioxidant enzymes in addition to mitochondrial proliferation.

While the circulating levels of RES in each treatment group is not known, it is interesting that the extent to which RES was able to induce changes in antioxidant enzymes and mitochondrial number was dependent on its route of administration. This perhaps suggests that the delivery modes were able to augment RES's transport and accumulation in the body. RES undergoes extensive chemical modifications in the small intestine following its ingestion, and is quickly metabolized (Sale et al., 2004). Despite its apparent low bioavailability, four weeks of chronic exposure to RES was sufficient to induce a number of changes in the observed enzymes. RES is a hydrophobic molecule, and it may be that interactions with serum proteins and lipids are able to increase localized concentrations, while circulating plasma levels remain low. In any case, administering RES in a high fat diet was highly effective in modifying antioxidant enzyme activities, and mitochondrial number. This property may be exploited to further increase the bioavailability of this polyphenol following ingestion.

In conclusion, administering RES in a high fat diet is capable of increasing enzymes involved in ROS metabolism in brain and heart. Most notably, RES in a high fat vehicle may represent a dietary means of achieving the protective effects of increased MnSOD levels in the brain.

Chapter 7: The Effects of Resveratrol and Phytoestrogens Provided in a Dietary Silicon Formulation on Antioxidant Enzymes in Mice

Hypothesis: The bioavailability of RES is very low. This presents a challenge in the design of dietary regimes aimed at capturing RES's beneficial effects. One way to overcome the challenge of RES's limited bioavailability is to maximize the dosage using supplements that may be added directly to foods. Silicon delivery systems may be used to encapsulate RES for delivery as a supplement. I hypothesize that a silicon delivery system will be an effective method to delivery RES and other ERbeta agonists.

Objectives: The objectives of this project were to examine antioxidant enzymes in brain, heart, and liver tissues of mice given RES, DPN, daidzein or coumestrol in silicon based dietary supplements.

Contributions:

I performed all experiments, statistical analysis and manuscript preparation.

7.1 Introduction

Attempts to demonstrate interactions between RES and various target molecules or pathways *in vitro* have been met with near universal success. As a consequence of this success RES has become a popular subject of biomedical research. One essential component of RES's molecular mechanism is an induction of the mitochondrial antioxidant enzyme MnSOD in an ERbeta dependent manner (Robb and Stuart, 2011; Chapter 2, Chapter 4). The upregulation of MnSOD underlies two of RES's important cellular activities: an inhibition of replicative cell growth and an ability to confer cytoprotection (Robb and Stuart, 2011; Chapter 2), which may ultimately give rise to RES's anticancer and neuroprotective effects *in vivo*.

Importantly, while the RES-stimulated induction of MnSOD occurs in multiple cell types *in vitro*, it is also observed in the brain tissue of mice fed RES in a high fat diet (Robb et al., 2008b; Chapter 6), and in skeletal muscle of RES supplemented mice given a normal diet (Jackson et al., 2011). Transgenic overexpression of MnSOD alone confers numerous health benefits including improved insulin sensitivity in mice (Boden et al., 2012), protection against tumour formation (Zhao et al., 2001), and enhanced neuroprotection against toxins such as MPTP (Klivenyi et al., 1998; Callio et al. 2005; Keller et al., 1998; Shan et al., 2007). The use of RES to realize the beneficial effects of MnSOD overexpression *in vivo* is thus an exciting potential application for this polyphenol.

RES is found naturally in some plant derived foods and beverages. Red wine is a rich dietary source of RES and its closely related structural analogues (*i.e.* piceid),

providing up to 14mg of these compounds per litre of wine depending on the grape varietal and viticulture practices (Dourtoglou et al., 1999; Naugler et al., 2007). However, achieving the dose of RES required for many of its physiological effects through diet alone is challenging, if not impossible. In humans positive effects on metabolic health are observed with a dose of 150mg/day RES in obese males (Timmers et al., 2012). This would require an intake of approximately 10L of red wine daily to achieve the necessary dose of RES. In mice a dose of 400mg RES /kg chow/day is required to see the reported improvements in aerobic exercise capacity and motor function (Lagouge et al., 2006). Reaching these very high levels of RES intake via diet alone is extremely unlikely, and its development into a dietary supplement is an important step in RES's further therapeutic use.

In addition to its relatively low abundance in the human diet RES is also rapidly metabolized following its ingestion and plasma and tissue levels of the pure compound are only a small fraction of the initial dose. While subcutaneous injection may provide an alternative means of delivery that can circumvent some chemical modifications made during digestion and absorption, dietary intake is more amenable to the long term supplementation regimens that are being investigated for RES. An interesting observation is that incorporation into a high fat diet is required for many of RES's effects, including its ability to enhance aerobic exercise capacity, improve insulin sensitivity and elevate levels of endogenous antioxidant enzymes (Baur et al., 2006; Lagouge et al., 2006; Robb et al., 2008b; Chapter 6). RES is hydrophobic, and readily dissolves into lipid based carriers. While the observed diet-specific effect may be reflective of RES's ability to correct the negative impact of a high fat diet, it is also

plausible that the delivery matrix shields RES from modifications made during metabolism and improves its dissolution in the digestive tract.

An unexplored strategy for the dietary delivery of supplemental levels of RES is the use of non-toxic silicon based drug carriers designed to shield RES from aqueous environments and protect it from degradation in the intestinal tract. Silicon is non-toxic, naturally occurring, and is widely used in a number of food products (*e.g.* soft drinks) and supplements (Sripanyakorn et al., 2009). Dietary delivery in a silicon matrix has been shown to enhance the absorption and plasma levels of drugs and compounds including insulin and ibuprofen (Salonen et al., 2005; reviewed in Anglin et al., 2008).
The hydrophobic core of the silicon carrier can accommodate high amounts of hydrophobic molecules (*i.e.* RES), while the hydrophilic exterior of the carrier shell is soluble in aqueous environments such as the lumen of the digestive tract. The release profile of drugs from a silicon carrier can also be adjusted for slow or fast dissociation, and can be regulated to control the location of release by pH dependent stability (reviewed in Anglin et al., 2008). The application of a silicon delivery system to realize the potential effects of RES have not been investigated *in vivo*.

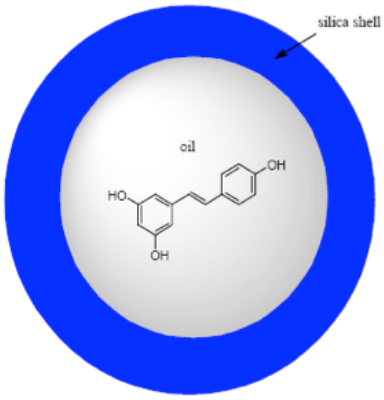


Figure 7.1. Silicon encapsulation of resveratrol.

In this study the ability of a dietary supplement of silicon encapsulated RES to affect antioxidant enzymes including MnSOD in mouse brain, heart and liver tissues was evaluated. RES is a phytoestrogen (Ghem et al., 1997; Bowers et al., 2000), and ERbeta is required for its *in vitro* effects on MnSOD (Chapter 4). To corroborate this *in vitro* mechanism of action *in vivo*, this feeding study was undertaken in both control mice and ERbeta null mice. RES is just one of many ER agonists capable of eliciting an induction of MnSOD *in vitro* (Chapters 4, Chapter 5). However, the other phytoestrogens identified to share RES's cellular activities (Chapter 5) have not been examined *in vivo*. Therefore, to explore the potential application of silicon encapsulation to the delivery of other ERbeta agonists, supplements containing DPN, coumestrol or daidzein were similarly investigated for their effects on antioxidant enzymes in mouse brain, heart, and liver tissue.

7.2 Methods:

7.2.1. Animal husbandry

For experiments involving silicon formulation 1 (SF1) C57Bl6, male retired breeders of approximately 6 months of age were purchased from Jackson labs (n=6). For experiments involving silicon formulation 2 (SF2) ER beta knockout, and control mice on a C57BL6 background were obtained from Taconic laboratories at 5 or 6 weeks of age (n=4-5 per treatment). All male mice were housed individually. Female mice were housed in groups of two or three of the same genotype. All mice were maintained in a temperature and humidity controlled environment on a 12 hour light/dark cycle. Mice were provided with water *ad libitum*. A finite amount of chow was provided daily. There was no significant decrease in body weight in any of the treatment groups during the experiment (data not shown).

7.2.3. Silicon Formulation Treatment

The silicon formulations used in this study were created and provided by Dr. Paul Zelisko (Department of Chemistry, Brock University). A preliminary experiment was performed with SF1 which was designed to provide a dose of 100mg/kg/day RES (n=6 male per treatment group). SF2 was designed to have improved drug release over SF1 at physiological pH, and provided a dietary dose of 150mg/kg RES (n=2 female, n=3 male), 80mg/kg coumestrol (n=3 female, n=2 male), 150mg/kg diadzein (n=3 female, n=3 male) or 10mg/kg DPN (n=2 female, n=2 male). The silicon formulations were mixed with approximately 5% w/w natural peanut butter (Kraft) to encourage mice to consume the treated chow. The control group received just the silicon formulation and peanut butter. All groups received only treated food which was provided daily. Mice

were on their respective diets for six weeks. All treatment protocols adhered to Brock University's animal care guidelines.

7.2.4. Tissue Harvesting:

At the end of their treatment, mice were sedated by isolfluorane inhalation then sacrificed by decapitation. Whole brain, liver, heart and kidney were removed and immediately snap frozen using liquid nitrogen, then transferred to -80°C for preservation.

7.2.5. Tissue Homogenization:

Frozen tissues were homogenized as described in Chapter 6.

7.2.6. Enzyme Activities:

Citrate synthase activity was assayed as outlined in Chapter 2.

Catalase activity was monitored as described in Chapter 2.

Glutathione peroxidase activity was measured as in Chapter 2.

The activities of MnSOD and CuZnSOD were measured using an in-gel assay as described in Chapter 6.

7.2.7 Western Blotting

Western blotting was performed as outlined in Chapter 2. 20μg of crude homogenate protein was used.

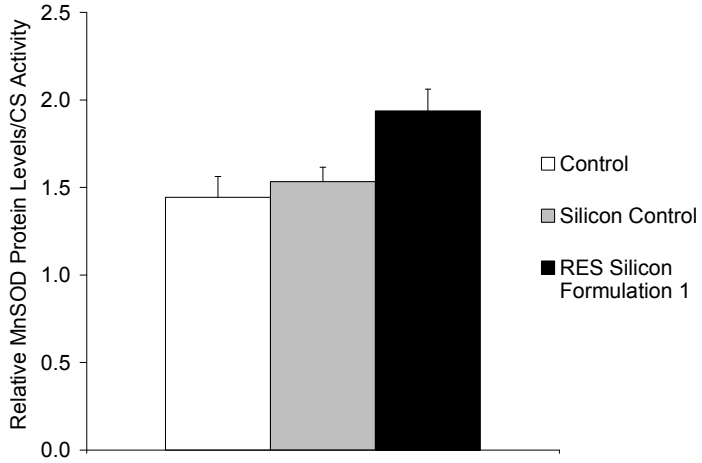
7.2.8. Statistical Analysis

Data collected were analyzed by ANOVA, followed by Tukey's post hoc test. Statistical analysis was performed using Systat. $p<0.05$ was taken as significant. Error bars represent SEM.

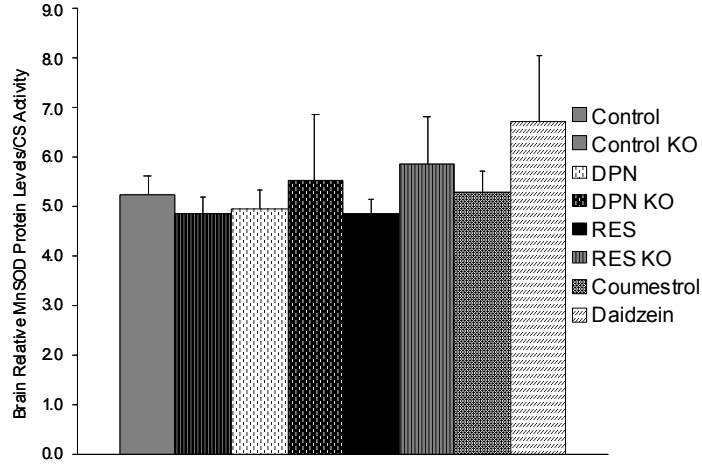
7.3 Results

An increase in MnSOD protein levels and activity has been previously reported with RES both *in vitro* (Robb et al., 2008) and *in vivo* (Robb et al., 2008b; Chapter 6; Jackson et al., 2011). To evaluate if a similar effect was observed when this compound was delivered to mice in a dietary silicon formulation the protein levels of MnSOD were measured in brain, liver, and heart tissue of mice given a RES supplemented or vehicle control diet for 6 weeks. The first silicon formulation (SF1) containing RES failed to invoke a significant induction of MnSOD protein levels in heart and liver tissue (data not shown). There was a trend toward increased MnSOD protein levels in the brain tissue of the RES SF1 treatment group, although this did not reach statistical significance (=0.06185) (Fig 7.2A). After the preliminary work with SF1, silicon formulation two (SF2) was designed to have improved drug release at neutral pH and an increased carrying capacity for RES and the other ERbeta agonists. SF2 was used to deliver RES, DPN, coumestrol or daidzein to wild type C57Bl6 mice. ERbeta null mice were given SF2 containing RES or the specific ERbeta agonist DPN as a positive control. In spite of the improvements made to SF2, these treatments failed to increase MnSOD protein levels in any of the examined tissues (Fig 7.2B, 7.2C, 7.2D). No significant differences in MnSOD protein levels were observed between the wildtype and ERbeta null control, or between control and treated groups.

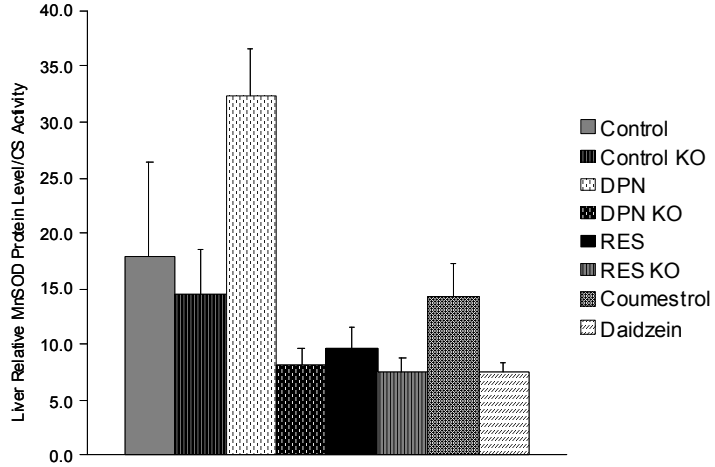
7.2 A


Relative MnSOD Protein Levels/CS Activity
2.5
2.0
1.5
1.0
0.5
0.0
Control
Silicon Control
RES Silicon Formulation 1

7.2 B


Brain Relative MnSOD Protein Levels/CS Activity
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Control
Control KO
DPN
DPN KO
RES
RES KO
Coumestrol
Daidzein

7.2 C


Liver Relative MnSOD Protein Level/CS Activity
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Control
Control KO
DPN
DPN KO
RES
RES KO
Coumestrol
Daidzein

7.2 D

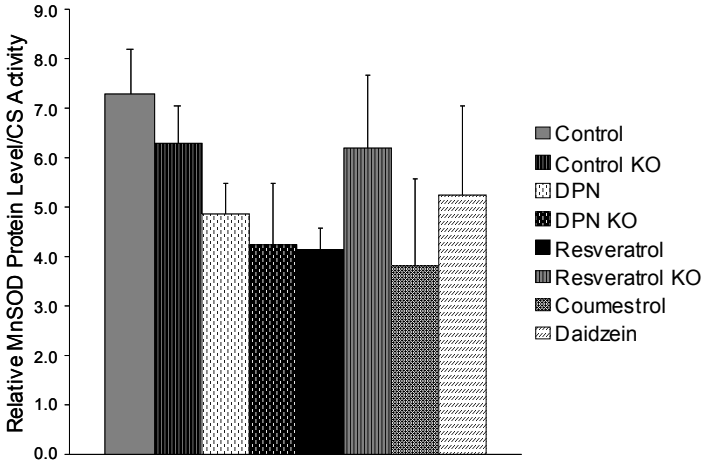
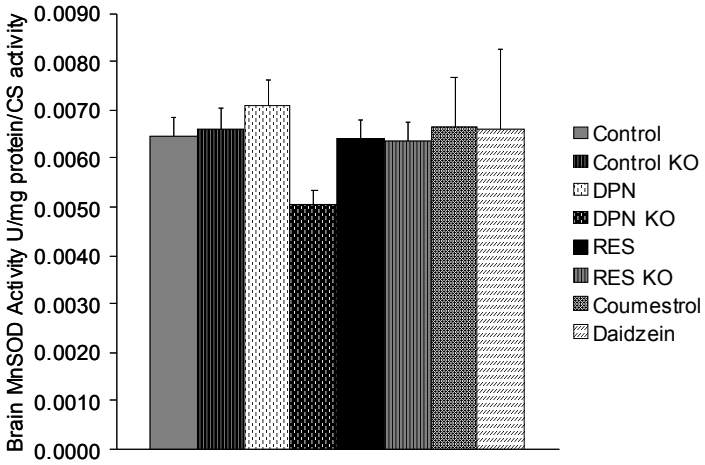


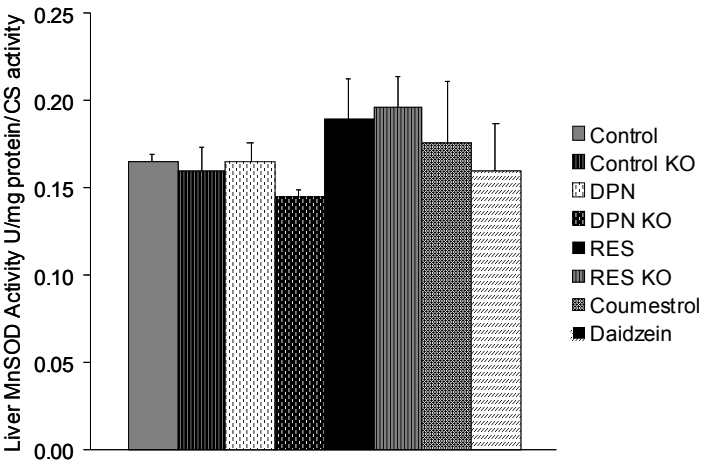
Figure 7.2. Relative MnSOD protein levels standardized to citrate synthase activity. A. Brain tissue of untreated, SF1 control and SF1-RES treated mice B. Brain tissue of wildtype and ERbeta null mice given SF2 treatments containing RES, DPN, Coumestrol or Daidzein C. Liver tissue of wildtype and ERbeta null mice given SF2 treatments containing RES, DPN, Coumestrol or Daidzein D. Heart tissue of wildtype and ERbeta null mice given SF2 treatments containing RES, DPN, Coumestrol or Daidzein. n=5-6. Values shown are means ± SEM of duplicate measurements of each homogenate.

MnSOD activity was recently shown to be regulated by acetylation (Zhu et al., 2012). To determine if MnSOD activity was increased independently of a significant change in protein level, activity was measured in the brain, heart and liver tissue of mice given the SF2 formulations. No significant differences were observed in MnSOD activity in the measured tissues of the SF2 treatment groups (Fig 7.3).

7.3 A


Brain MnSOD Activity U/mg protein/CS activity
0.0090
0.0080
0.0070
0.0060
0.0050
0.0040
0.0030
0.0020
0.0010
0.0000
Control
Control KO
DPN
DPN KO
RES
RES KO
Coumestrol
Daidzein

7.3 B


Liver MnSOD Activity U/mg protein/CS activity
0.25
0.20
0.15
0.10
0.05
0.00
Control
Control KO
DPN
DPN KO
RES
RES KO
Coumestrol
Daidzein

7.3 C

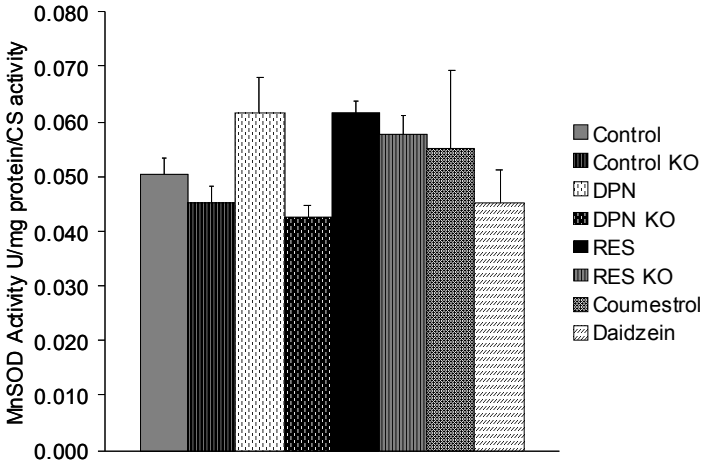
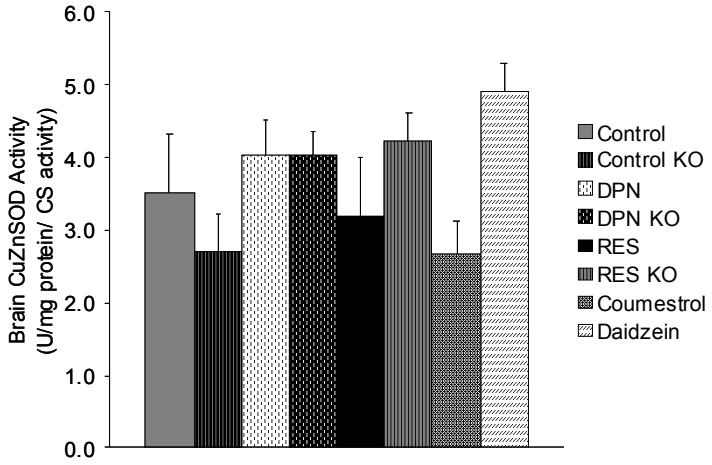


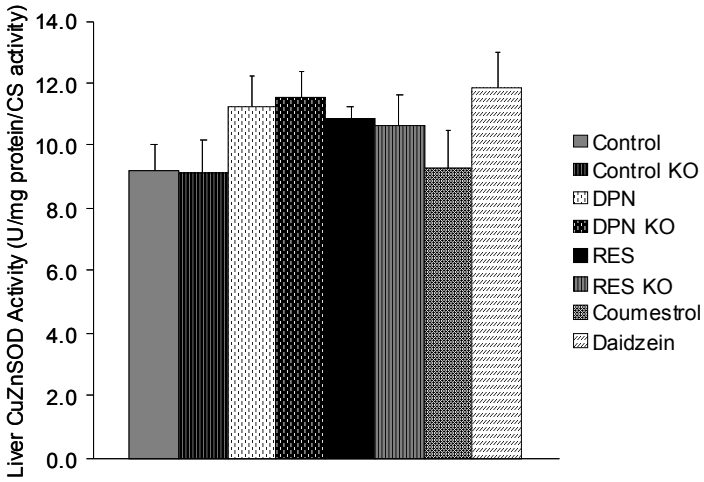
Figure 7.3. MnSOD Activty standardized to CS activity in wildtype and ER beta null mice.
A. brain tissue, B. liver tissue and C. heart tissue of wildtype or ERbeta null mice given SF2 treatments containing RES, DPN, coumestrol or daidzein. n=5-6. Values shown are means ± SEM of duplicate measurements of each homogenate.

In addition to its presence in the mitochondrial matrix superoxide may also exist in the cytosol. To determine if the SF2 was affecting the activity of the cytosolic superoxide dismutase, CuZn superoxide dismutase activity levels were measured in liver, heart and brain tissue. No significant differences in CuZn superoxide dismutase activity were observed across the experimental groups.

7.4 A


Brain CuZnSOD Activity (U/mg protein/ CS activity)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Control
Control KO
DPN
DPN KO
RES
RES KO
Coumestrol
Daidzein

7.4 B


Liver CuZnSOD Activity (U/mg protein/CS activity)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Control
Control KO
DPN
DPN KO
RES
RES KO
Coumestrol
Daidzein

7.4 C

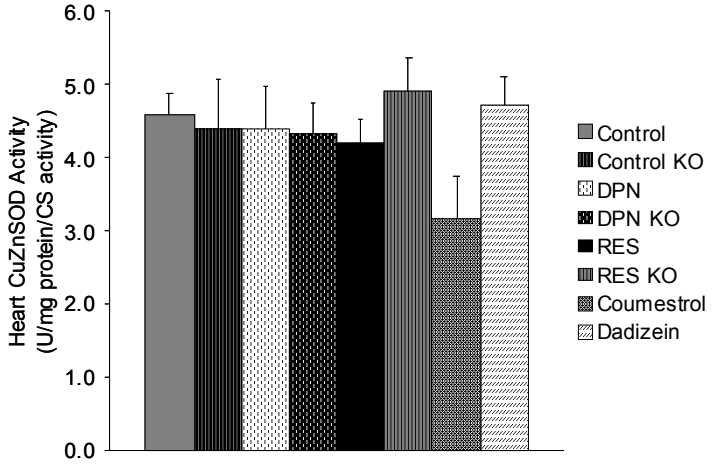
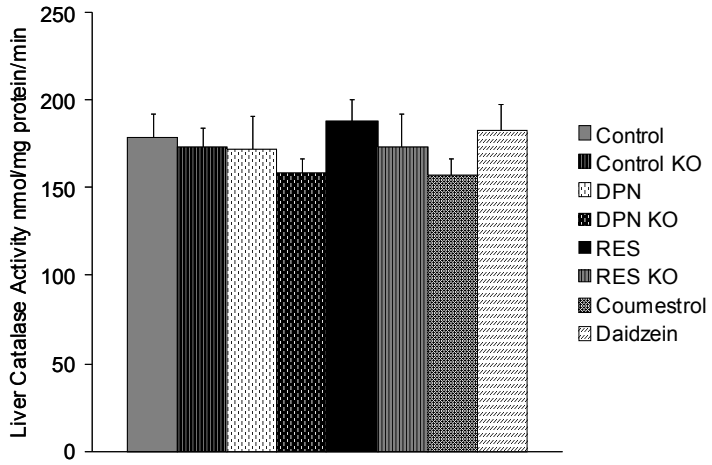


Figure 7.4. CuZnSOD Activty in wildtype and ER beta null mice.
A. brain tissue, B. liver tissue and C. heart tissue of wildtype or ERbeta null mice given SF2 treatments containing RES, DPN, coumestrol or daidzein. n=5-6. Values shown are means ± SEM of duplicate measurements of each homogenate.

ROS beyond the superoxide anion contribute to the redox status of the intracellular milieu. Elevated concentrations of intracellular hydrogen peroxide have been associated with the development of metabolic disorders including insulin resistance (reviewed in Henriksen et al., 2011), which is improved with RES treatment in both mice and obese humans (Baur et al., 2006; Timmers et al., 2012). To evaluate the SF2 treatment's effects on hydrogen peroxide metabolism activity measurements were made for two antioxidant enzymes that catalyze its removal: catalase and glutathione peroxidase. There were no significant differences in the activity of catalase in liver and heart tissue from the control and ER beta null mice fed the SF2 treatments. Catalase activity was not present at detectable levels in brain homogenates.

7.5 A



7.5 B

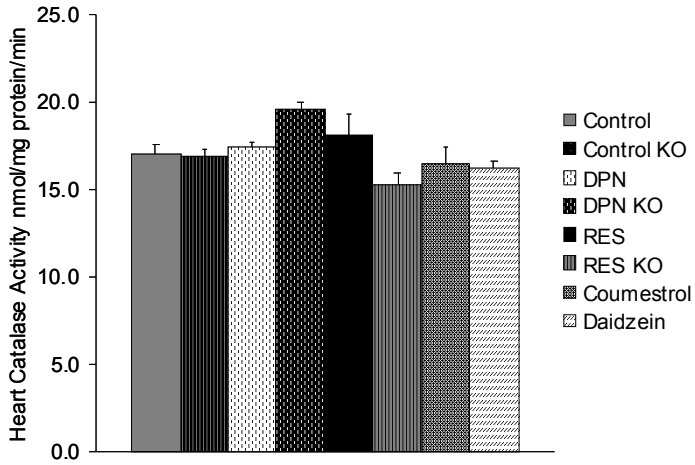
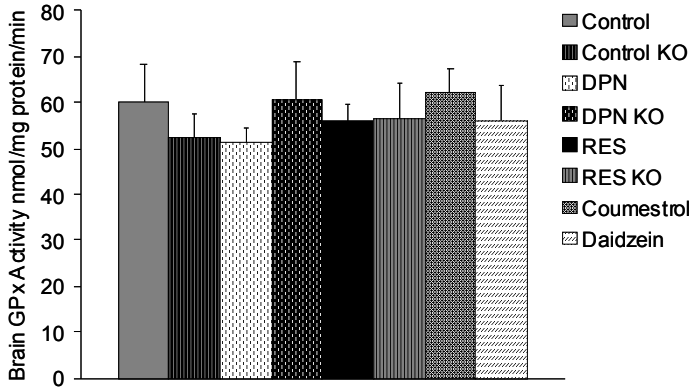


Figure 7.5. Catalase Activity in wildtype and ER beta null mice.
A. liver tissue and B. heart tissue of wildtype or ERbeta null mice given SF2 treatments containing RES, DPN, coumestrol or daidzein. n=5-6. Values shown are means ± SEM of duplicate measurements of each homogenate.

Glutathione peroxidase uses intracellular glutathione to reduce hydrogen peroxide to water. The resultant oxidized glutathione is subsequently reduced by the enzyme Glutathione reductase. The activities of both enzymes were measured in liver, heart and

brain tissue of wildtype and ER beta null mice given the SF2 formulations. There were no significant differences in enzyme activity between any of the experimental groups.

7.6 A



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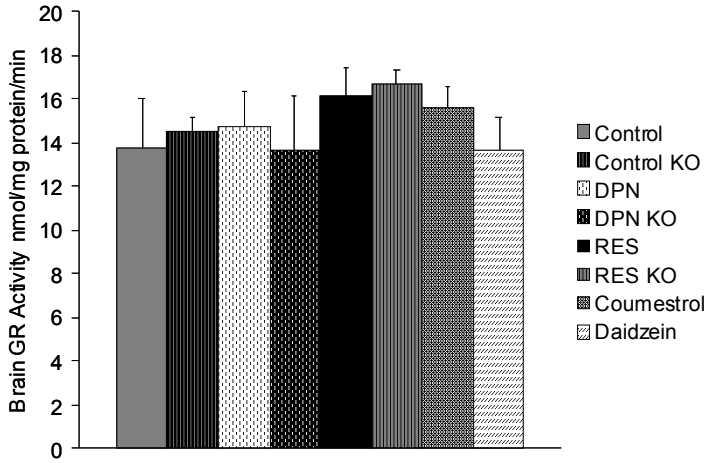

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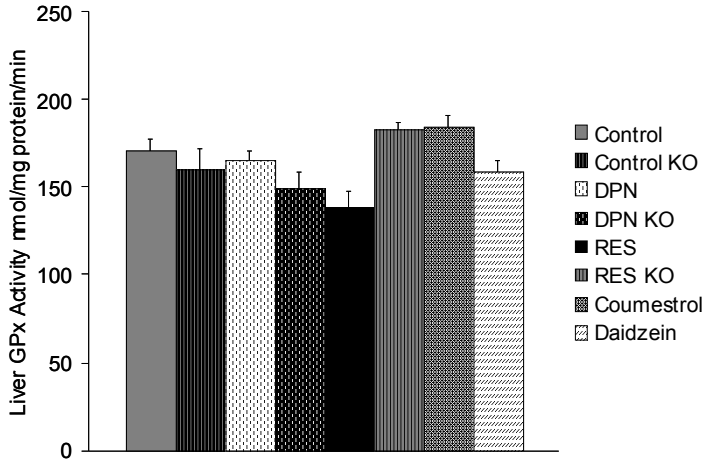

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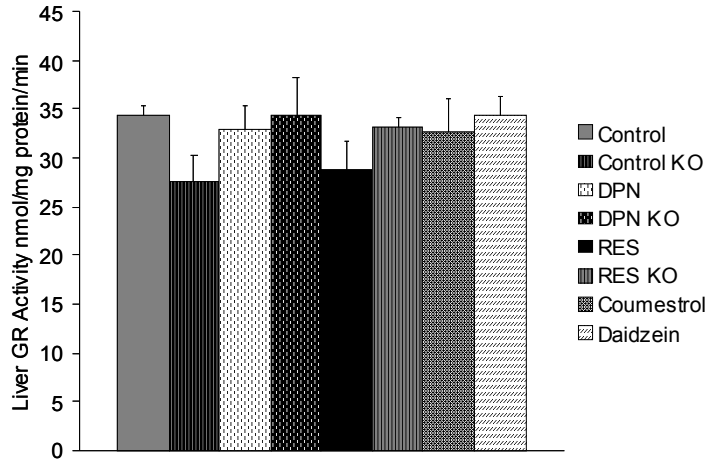
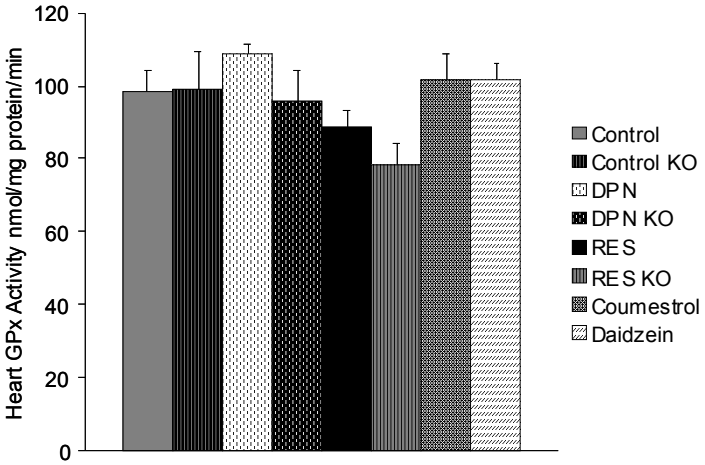

Liver GR Activity nmol/mg protein/min
45
40
35
30
25
20
15
10
5
0
Control
Control KO
DPN
DPN KO
RES
RES KO
Coumestrol
Daidzein

7.6 E


Heart GPx Activity nmol/mg protein/min
120
100
80
60
40
20
0
Control
Control KO
DPN
DPN KO
RES
RES KO
Coumestrol
Daidzein

7.6 F

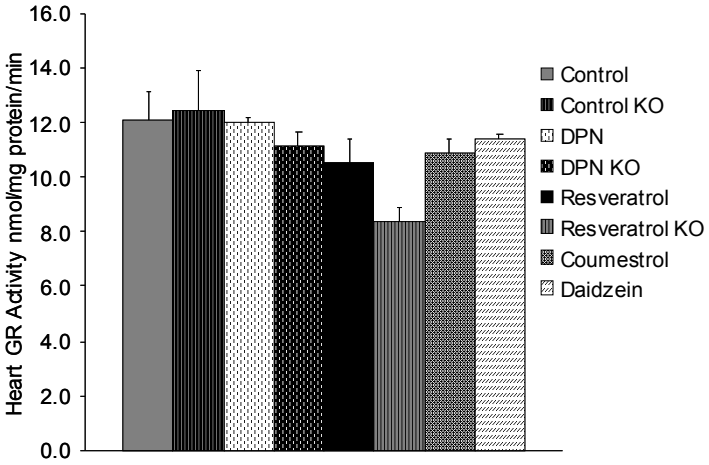


Figure 7.6. Glutathione peroxidase and glutathione reductase activty in wildtype and ER beta null mice.

A. Glutathione peroxidase activity in brain tissue, B. Glutathione reductase in brain tissue, C. Glutathione peroxidase in liver tissue, D. Glutathione peroxidase in liver tissue, E. Glutathione peroxidase in heart tissue, F. Glutathione reductase in heart tissue of wildtype or ERbeta null mice given SF2 treatments containing RES, DPN, coumestrol or daidzein. n=5-6. Values shown are means ± SEM of duplicate measurements of each homogenate.

7.4 Discussion:

Many of RES's purported effects on human health are consistent with an induction of MnSOD (*i.e.* anticancer and neuroprotective effects), and this phytoestrogen may therefore provide a means to capture the positive effects associated with MnSOD overexpression *in vivo*. A limitation of RES's use for this purpose is its very low bioavailability, and its relatively low abundance in the diet. This study was undertaken to determine if encapsulation within silicon nanoparticles could be used to effectively deliver RES as a dietary supplement to induce MnSOD expression *in vivo*.

Within the silicon nanoparticles exists a hydrophobic interior that provides a stable enivornment for non-polar molecules such as RES, while the outside surface of the nanoparticles is hydrophilic in nature and therefore soluble in aqueous environments. These structural characteristics permit the dietary delivery of a greater amount of water-insoluble compounds. The rate of drug release can be regulated by surface chemistry and the pore size of the silicon carrier (Kilpelainen et al., 2009). Recently, Tsai and colleagues (2011) generated a meso porous silicon carrier and tested its ability to deliver RES to cultured cells. The meso porous silicon carrier improved the growth inhibitory effect of RES in HeLa and Chinese hamster ovarian (CHO) cell lines. However, the effectiveness of the encapsulation used here *in vitro* was not explored.

The treatments provided here failed to elicit an induction of MnSOD in brain, heart and liver tissue of mice on a chronic treatment regime. While the circulating levels of RES or the other ERbeta agonists are unknown, *in vitro* studies with the SF1 and SF2 formulation predicted that the polyphenol would be released from the carrier after absorption in the small intestine due to the change in pH moving from the stomach to

small intestine (P.M. Zelisko, unpublished data). The silicon nanoparticles were designed to maintain their integrity in the low pH environment of the stomach, and to dissociate and release RES in the neutral pH environment of the small intestine. The doses chosen for the dietary ERbeta agonists RES, and coumestrol were at a supplement level, and were similar to, or above, treatment levels previously reported in the literature (Lagouge et al., 2006; Ueda et al., 2012). In humans, plasma concentrations of coumestrol from food are in the low nanomolar range (Mustafa et al., 2007). Daidzein, which is a component of soy-based foods may reach concentrations as high as 10μM (Adlercreutz et al., 1993). Given the high doses of these compounds used in this experiment it is unlikely that these compounds were not present in plasma at appreciable levels.

In other experiments involving dietary RES administration, significant effects on metabolism and aerobic exercise endurance are observed when RES is added to diets with fat contents equal to or greater than 40%, but not when RES is added to a standard composition diet (Baur et al., 2006; Lagouge et al., 2006). Similarly, RES given in a high fat diet significantly increases brain antioxidant enzyme activities, while the same dose given in a standard mouse diet does not have a significant effect (Robb et al., 2008b; Chapter 6). An often-overlooked factor in these studies is the fat source used to augment the animal diet. The use of oils high in monounsaturated and polyunsaturated fatty acids to increase the fat component of the diet can have substantial physiological effects (Lau et al., 2010) that may alter the response to RES. It may also be the case that the high fat diet impacts the intestinal absorption and metabolism of RES, which is a lipophilic compound. RES undergoes extensive chemical modification in the intestinal

tract that enables its degradation (Kuhnle et al., 2000), and its inclusion in a high fat diet may alter the degree to which it is modified. This has not yet been thoroughly investigated.

In light of these observations of an apparent requirement for delivery in a high fat diet to elicit many of RESs effects in vivo, it would seem possible that dietary strategies could be used to improve RES's bioavailability in humans. However to date, little research exploring this idea has been published. Vitaglione and colleagues (2005) observed that circulating levels of RES following red wine intake were not influenced by the macromolecule composition of an accompanying meal in healthy human subjects (Vitaglione et al., 2005). While this result does not support the argument that diet composition influences RES uptake, the strength of any conclusions based on this study is limited by the fact that the plasma concentrations of RES detected in this study were extremely low. Understanding the impact of diet composition on RES's effects is important for its potential to inform nutritional strategies and the development of new delivery methods with the goal of increasing bioavailability, and will be an area of ongoing research.

A unique aspect of this study was the use of both male and female mice. This is an important consideration in the development of pharmaceuticals and dietary supplements that are intended for use in both sexes. However, the use of both male and female mice may also contribute to increased variability in treatment response within experimental groups. Previous studies demonstrating RES's physiological effects *in vivo* have employed almost exclusively male mice, or ovariectomized females (*e.g.* Baur et al., 2006; Lagouge et al., 2006; Park et al., 2012). RES, and the other phytoestrogens tested,

are likely to exert different effects on males and females. In females, physiological response to RES and other phytoestrogens may be influenced by changes in endogenous hormone levels that occur with the estrous cycle. Estrogen increases MnSOD protein levels (Borras et al., 2010; Pedram et al., 2006), and in mice females have greater MnSOD activity levels than males (Vina et al., 2006). In this study, the low number of individuals of each sex within treatment groups (n=2-3) did not provide a large enough cohort in which to examine the effect of this variable in response to treatment. The sex-specific effects of both RES and other phytoestrogens require more detailed investigation.

As there was no statistically significant difference in wildtype animals treated with RES, the inability of the RES or DPN containing formulations to elevate MnSOD levels *in vivo* does not further an appreciation for the role of this receptor in RES's effects. Analysis of the wildtype and ERbeta null animals receiving only the control formulation did not reveal any statistically significant differences in any of the antioxidant enzymes measured between groups, suggesting that the basal levels of antioxidant enzymes are unchanged by deletion of the ERbeta. Further investigation of RES effects in *in vivo* in the context of ERbeta deletion is required.

In conclusion, this study failed to observe a significant effect of a six week treatment with RES, the ERbeta agonist DPN, or the dietary phytoestrogens coumestrol and daidzein, in a dietary silicon formulation on antioxidant enzymes in wildtype and ERbeta null mice. Further studies that include measures of drug metabolite levels in plasma, and an increased sample size are required.

Chapter 8. General Discussion

RES is a phytoestrogen present at appreciable concentrations in red wines that elicits a diverse array of phyioslogical effects in animals, including potent anticancer and neuroprotective effects. On a cellular level, it is plausible that RES's ability to slow proliferative cell growth and to impart protection against stress induced cell death may give rise to its positive effects on health. However, the proximal targets of RES responsible for these two cellular effects have not been clearly established.

The goal of this thesis work was to delineate the cellular mechanisms responsible for two of RES's fundamental effects in mammalian cells: an inhibition of proliferative growth, and cytoprotection. To this end the mitochondrial antioxidant enzyme MnSOD was established as a critical target of RES *in vitro* and *in vivo* that is required for RES's influence on these cellular properties. The discovery that MnSOD is induced by RES treatment (Robb et al., 2008a) has been corroborated by other researchers who have repeated this observation in a variety of cell types and tissues. For example, a RES stimulated increase in MnSOD levels is observed in cultured cardiomyocytes, neuroblastomas (SK-N-BE), a hippocampal neuronal cell line (HT22), coronary arterial endothelial cells, and pheochromocytoma cells (PC6.3) (Movahed et al., 2012; Albani et al., 2009; Fukui et al., 2010; Ungvari et al., 2009; Kairisalo et al., 2011). *In vivo*, RES has been reported to increase MnSOD in skeletal muscle, hematopoietic stem cell populations, and dopaminergic neurons in mice (Jackson et al., 2011; Zhang et al., 2013; Mudo et al., 2012). Thus, the induction of MnSOD by RES is a robust observation, and MnSOD is a well established target in RES's molecular mechanism in mammals.

8.1 The importance of MnSOD

The identification of MnSOD as a proximal target of RES is interesting in light of its central role in many aspects of cell physiology. MnSOD activity has been implicated in the regulation of proliferative cell growth and stress resistance. Overexpression of MnSOD in embryonic fibroblasts promotes the transition of actively mitotic cells into quiescence (Sarsour et al., 2008), and can significantly slow the replication rate of transformed cell lines (Li et al., 1998; Weydert et al., 2006). MnSOD overexpression also imparts cytoprotection against a range of stressors (Kliveny et al., 1998; Motoori et al., 2001), and protects against cell death in models of ischemia and neurodegeneration (Keller et al., 1998; Dumont et al., 2009). As would be predicted wtih MnSOD overexpression, RES treatment reduced proliferative growth and conferred cytoprotection in cultured myoblasts, fibroblasts, neuroblastoma and prostate cancer cell lines. When the MnSOD induction was prevented, RES failed to elicit a significant change in these characteristics (Robb and Stuart, 2011; Chapter 2; Chapter 4; Chapter 5). Thus, RES treatment mimics the effects associated with MnSOD overexpression in cultured cells.

Interestingly, MnSOD is a downstream target of multiple signaling pathways known to influence animal health and lifespan. For example, MnSOD levels are increased in response to rapamycin, a pharmacological inhibitor of mTOR that extends lifespan in mice (Iglesiase-Bartolome et al., 2012; Harrison et al., 2009). MnSOD has also been identified as a downstream target of the transcription factor FOXO3a which is regulated by the insulin/insulin-like growth factor-1 pathway, a highly conserved pathway shown to influence both cellular stress resistance and lifespan in a variety of

animal models (Kops et al., 2002, Baba et al., 2005; Yamamoto et al., 2005; reviewed by Longo and Fabrizio, 2002).

The Free Radical Theory of Aging posits that the accrual of cellular oxidative damage underlies the development of age-related disease and limits animal lifespans (Harman 1956; Harman 1972). Perhaps in light of this long standing hypothesis the role of MnSOD in these various contexts has been interpreted solely as a mechanism to reduce oxidative damage. However, a substantial body of evidence exists in dispute of the predictions of the Free Radical Theory of Aging and the consequences of oxidative damage generally. In *C. elegans*, exposure to the mitochondrial superoxide generator paraquat actually increases lifespan (Yang and Heikimi et al., 2010), and increased oxidative damage arising from the deletion of MnSOD and glutathione peroxidase 1 does not negatively impact on the lifespan of adult mice (Zhang et al., 2009). Similarly, dietary supplementation with small molecule antioxidants capable of reducing intracellular ROS levels does not extend lifespan in mice (*e.g.* Strong et al., 2012). It therefore seems unlikely that a simple reduction in oxidative damage is sufficient to account for the many effects attributed to MnSOD.

In contrast to their propensity to non-discriminately oxidize macromolecules at high concentrations, at low concentrations ROS may regulate signaling pathways via the oxidation of redox sensitive cysteine residues found in various enzymes and regulatory factors, including those that participate in control of the cell cycle (see Burhans and Heintz, 2009 for review). The only enzymatic activity that has been ascribed to MnSOD is the dismuation of the membrane impermeable superoxide anion to produce hydrogen peroxide, which is capable of diffusing across biological membranes and may act to relay

information relating to mitochondrial redox status throughout the cell. The ability of RES to elicit an induction of MnSOD was maintained in ρ^0 human prostate cancer cells (PC3) that are unable to respire and therefore produce minimal levels of mitochondrial ROS. However, in the absence of mitochondrial superoxide production the RES simulated induction of MnSOD was not accompanied by a reduction in proliferative cell growth (Chapter 4). This suggests that ROS metabolism is required for both RES and MnSOD to affect the cell cycle. Population doubling time in the ρ^0 PC3 cells was substantially greater than in wildtype PC3s. Significantly slowed cell division has also been reported in ρ^0 human cervical cancer cells (HeLa) (Schauen et al., 2006). These data support the hypothesis that ROS arising from oxidative phosphorylation promotes proliferative cell growth, and that the MnSOD mediated modulation of ROS levels underlies the ability of this enzyme to regulate proliferative cell growth.

While the specific regulatory factors that were impacted by elevated MnSOD levels were not identified here, previous research has implicated cyclin D1 and p27 as being subject to redox sensitive modifications that regulate cell cycle progression (Menon et al., 2003; Menon et al., 2007). Further study of the potential sites of redox modification and their relation to superoxide mediated signaling is required to understand this relationship.

8.2 Mitochondria as a General Target of Resveratrol

Many of the observations made with RES or estrogen treatment generally can be attributed to changes in mitochondrial physiology. Mitochondria exist as dynamic structures that fluctuate between a population of segmented, ovoid-shaped fragments and a highly connected, tubular reticulum. Interestingly, the inhibition of proliferative cell

growth and cytoprotection that was concurrent with the RES-stimulated induction of MnSOD could be phenocopied by a pharmacological inhibitor of mitochondrial fission (Chapter 3). In fact, many parallels exist between MnSOD overexpression and a highly fused mitochondrial network. Both mitochondrial hyperfusion and MnSOD overexpression can increase mitochondrial membrane potential, and reduce susceptibility to apoptotic cell death (reviewed in Youle and van der Bliek, 2012; Silva et al., 2005; Kokoszka et al., 2001). A hyperfused reticulum is also able to impede progression through the cell cycle, which is similar to what is observed with MnSOD overexpression (Mitra et al., 2009; Weydert et al., 2006; Li et al., 1998). Mitochondria fusion is promoted in response to hydrogen peroxide and increased concentrations of oxidized glutathione (Shutt et al., 2012), which may occur in localized regions as a product of MnSOD activity. This redox change regulated by MnSOD, and leading to increased fusion may provide a molecular mechanism to account for the ability of mitochondria to direct cellular proliferation independently of other regulatory cell cycle factors. Surprisingly, changes in mitochondrial connectivity and the accompanied redox modifications to fission and fusion proteins have not yet been studied in relation to MnSOD levels.

This work is the first to investigate the effect of RES on mitochondrial morphology. However, there is some experimental evidence to support a role for estrogens in regulating mitochondrial dynamics. In astrocytes, estrogen treatment significantly increases the expression of fusion and fission related genes (Arnold et al., 2008). In skeletal muscle, estrogen stimulates the transcription of mitofusion 2 (Liesa et al., 2008), an important protein involved in the mechanism of fusion. In contrast,

ovariectomy in female mice results in an increase in the expression of the fission protein Drp1 and a reduction in the expression of the fusion protein OPA1 (Yao et al., 2011). While previous research has not examined the individual contribution of ERalpha or ERbeta, in this study RES and DPN stimulated mitochondrial fusion in cultured myoblasts in an ERbeta dependent manner, suggesting that it is this receptor that plays an important role in mediating the fusion effects of estrogen (Chapter 3). Further studies are needed to clarify the details of this mechanism.

8.3 Upstream of the MnSOD Induction

The cellular mechanisms of RES have been a controversial subject amongst researchers. The importance of RES's actions as a phytoestrogen has been overshadowed by more recent, and contentious, claims that RES is a direct activator of SirT1. Data collected in this thesis supports the hypothesis that RES's effects on proliferative cell growth and stress resistance are directly related to its actions as a phytoestrogen.

8.3.1 No Evidence for a Direct Involvement of Sirtuins

Many of RES's biological activities have been attributed to a direct stimulation of mammalian sirtuin 1 (SIRT1) activity (*i.e.* Howitz et al., 2003; Wood et al., 2004; Valenzano et al., 2006). However, it is important to note that the data collected in the *in vitro* experiments used to demonstrate an allosteric activation of SIRT1 by RES are greatly affected by an artefactually high signal that arises from RES's interaction with the fluorescent reporter system used (Pacholec et al., 2010; Beher et al,. 2009; Kaeberlein et al., 2005; Borra et al., 2005). In spite of the evidence against a direct stimulation of SIRT1 activity, the purported role of SIRT1 in RES's molecular

mechanism continues to be debated within the scientific community. In this thesis, two experimental approaches were taken to evaluate the contribution of SIRT1 to the RES-induced increase in MnSOD: 1) Chemical inhibition of SIRT1 activity using sirtinol 2) SirT1 null mouse embryonic fibroblasts. In both instances RES's effects on MnSOD persisted (Chapter 2; Chapter 4). The absence of SIRT1 reduced the activity of citrate synthase and negatively affected cellular stress resistance, perhaps suggesting that the deletion of SIRT1 is generally detrimental to cell physiology. Although there was no evidence that RES's effect on MnSOD and in turn proliferative cell growth and stress resistance was dependent upon an activation of SIRT1, acetylation may still play a tangential role in RES's effects on MnSOD.

MnSOD activity is regulated by acetylation. Deacetylation catalyzed by the mitochondrial enzyme SIRT3 increases the activity of MnSOD (Tao et al., 2010; Zhu et al., 2012), and SIRT3 levels are increased by RES treatment *in vitro* (Hayakawa et al., 2012). In this thesis the role of SIRT3 in the cellular mechanism of RES was not explored. However, changes in MnSOD protein levels were closely paralleled by changes in enzyme activity, which does not support a role for post translational modification as a singular means of increasing MnSOD activity over the relatively long durations (several days) of these experiments. Overall, while it is plausible that deacetylation via sirtuins may participate in the biological activities of RES it is unlikely to play a directive role in RES's effect on MnSOD.

8.3.2 Estrogen Receptors and Resveratrol

The structural similarity between the RES, estrogen, the ERbeta agonist DPN and the synthetic estrogen diethylstilbestrol is striking (Fig. 8.1). Not surprisingly, there is

substantial overlap in the physiological effects of these molecules. RES has been previously identified

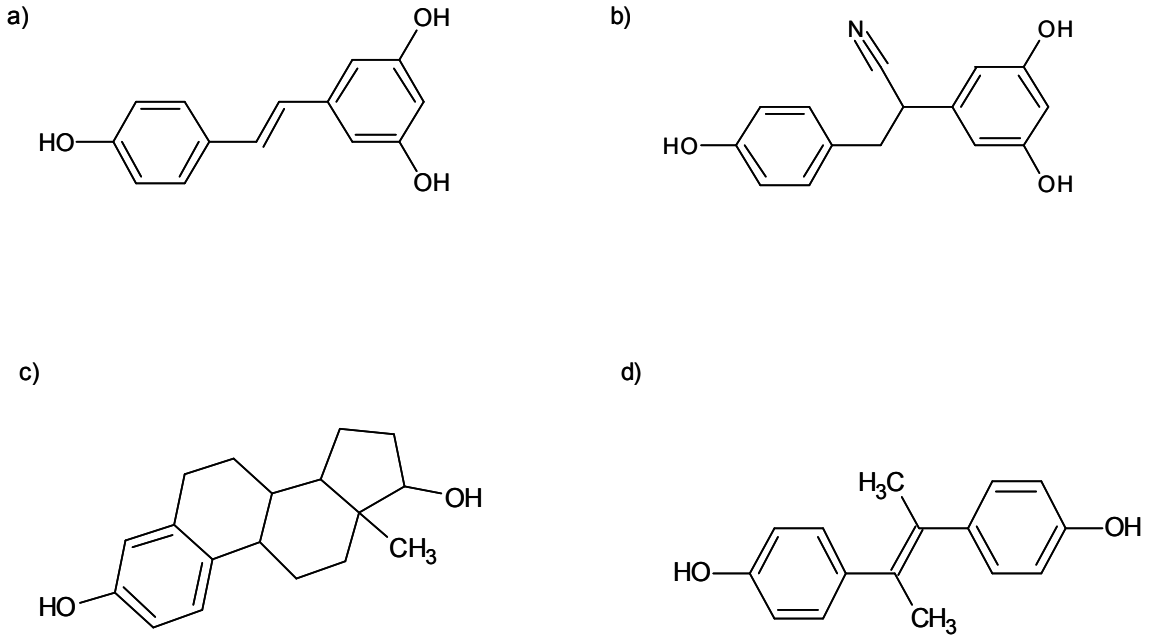


Figure 8.1. Resveratrol is structurally similar to estrogens.
Chemical structure of a) Resveratrol b) DPN c) 17β-Estradiol and d) Diethylstilbestrol

as an ER agonist (Gehm et al., 1997). In this work, the antiproliferative and cytoprotective effects of RES were phenocopied by both estradiol and the specific ERbeta agonist DPN, were inhibited by the estrogen receptor antagonist ICI182780, and were absent in cell lines generated from ERbeta null mice (Chapter 4, 5). Thus ERs, and in particular ERbeta, play an important role in RES's effect on MnSOD, proliferative cell growth and stress resistance.

Estrogens exert a variety of effects on mitochondrial function, including a stimulation of mitochondrial biogenesis that can be inhibited with the estrogen receptor

antagonist ICI182780 (*e. g.* Mattingly et al., 2008; reviewed in Klinge 2008; Chen et al., 2009). In contrast, a decrease in estrogen levels resulting from ovariectomy in female mice is concurrent with a reduction in the levels and activities of mitochondrial respiratory chain proteins (Yao et al., 2010; Yao et al., 2011). Similar to estrogen, RES stimulates mitochondrial biogenesis under a variety of experimental conditions. *In vitro* RES treatment stimulates mitochondrial biogenesis, and an upregulation of specific mitochondrial proteins occur in cultured human coronary arterial endothelial cells (Csizar et al., 2009). A stimulation of mitochondrial biogenesis with RES treatment has also been demonstrated *in vivo*. In mice a high fat diet supplemented with RES increases mitochondrial abundance, mtDNA copy number and citrate synthase activity in skeletal muscle (Lagouge et al., 2006). In this work, an increase in citrate synthase activity was observed with RES, estradiol and DPN treatment in fibroblasts, myoblasts, prostate cancer cells, but was not observed with the ERalpha agonist PPT.

A second similarity between estrogens and RES is their ability to elevate MnSOD levels *in vitro* and *in vivo*. In cultured vascular smooth muscle cells estrogen treatment significantly increases MnSOD activity, and this is accompanied by a reduction in proliferative cell growth (Sivritas et al., 2011). In rats estrogen treatment increases MnSOD levels in mitochondria isolated from brain tissue (Razmara et al., 2007), and a downregulation of MnSOD is observed in vascular tissue of ovariectomized mice (Strehlow et al., 2003). Estrogens also exert rapid and direct effects on MnSOD activity. In mitochondria isolated from breast cancer cells (MCF7) estrogen treatment rapidly, and significantly increases MnSOD activity through an unknown pathway that requires ERs (Pedram et al., 2006). As discussed above MnSOD is an essential target of RES that is

required for its growth inhibitory and cytoprotective effects, and the parallel to estrogen's effects on this enzyme further supports the hypothesis that ERs are important component of RES's molecular mechanism.

Three ERs have been identified to date: ERalpha, ERbeta and the non-genomnic G-protein coupled estrogen receptor. In this work, ERbeta was identified as being necessary for RES to affect proliferative cell growth and cytoprotection (Chapter 4, Chapter 5). While the exact role of ERbeta in cell physiology remains to be determined, the existing data suggests that ERbeta is generally associated with an inhibition of cell proliferation. For example, DPN inhibits growth of the murine colon cancer cell line MC38 *in vitro* (Motylewska et al., 2009), and of cells in the colon and small intestine of ovariectomized rats (Schleipen et al., 2011). Previous research has reported that RES is capable of binding to both ERalpha and ERbeta with equal affinity (Bowers et al., 2000), however a recent computational study of phytoestrogens and ER binding preferences indicates that RES should bind preferentially to ERbeta over ERalpha when the affinity of these compounds for the agonist and antagonist receptor conformations were taken into consideration (Yuan et al., 2011). Thus, RES may exert its cellular effects via ERbeta agonism.

An important consideration when interpreting these data is that the response to ERalpha and ERbeta binding is highly dependent on the presence of other factors that can act as coactivators or repressors (Zhao et al., 2010). In this study ERbeta null cell lines were generated as a tool to isolate the effects of this ER. Unfortunately, it was not possible to quantify the protein level of ERbeta in these experiments, as the currently available commercial antibodies to murine ERbeta do not show the specificity necessary

to draw conclusions about its protein levels and localization in cell and tissue samples (Snyder et al., 2010). This technical limitation prevented an evaluation of the changes in ERbeta levels in response to chronic RES treatment both *in vitro* and *in vivo*. Understanding the role of ERbeta in physiology and improving the current techniques to quantify its protein levels is an essential step in realizing its role in the biological activities RES.

RES's ability to act as an ER agonist appears to underlie its effects on MnSOD, and in turn on proliferative cell growth and stress resistance. This is an exciting observation as many other dietary compounds that are ER agonists, and specific ERbeta agonists, may be able to elicit the same positive effects on health as RES.

8.4 Resveratrol's Biological Activities are Shared by Other Phytoestrogens.

RES has become a much-celebrated bioactive component of red wine for its beneficial effects on many human ailments. Given that RES's mechanism of action may be related to its ability to act as an estrogen agonist, it seems unlikely that the biological activity of RES is truly unique. A more plausible hypothesis is that this compound is simply the most studied of a very large group of bioactive compounds found in red wines with similar and complimentary functions in mammalian cells. The data collection presented here supports this hypothesis, as several structural analogues of RES (*i.e.* pterostilbene, piceid) and phytoestrogens (*i.e.* genistein, coumestrol) similarly stimulated an upregulation of MnSOD leading to reduced proliferative growth and enhanced cytoprotection. A shared molecular mechanism between several phytoestrogens raises the possibility that limitations relating to RES bioavailability could be circumvented through the additive, or even synergistic effect of multiple

phytoestrogens. While investigations aimed at uncovering the biological activities of phytoestrogens typically involve experiments with the isolated compound this does not necessarily reflect how these compounds are consumed in the diet, which is usually in complex matrix of many biologically active molecules. There is some experimental evidence to suggest that a combination of phytoestrogens at very low concentrations may elicit biological activities in mammalian cells. A mixture of 12 different dietary phytoestrogens to provide a total concentration of 1μM significantly stimulated estrogen production in cultured breast cancer cells (MCF7) (Taxvig et al., 2010). Similarly, a combination of the phytoestrogens genestein, quercetin and biochanin A yields a synergistic effect on the inhibition of prostate cancer cell proliferation (PC3, DU-145 and LNCaP) (Kumar et al., 2011). The potential for additive or synergistic effects of polyphenols was not investigated here. However, with the identification of a shared molecular mechanism the potential for additive, or even synergistic effects seems promising.

8.5 Resveratrol Concentrations *in vitro*: Important Considerations

Strategies to elucidate the cellular mechanisms that give rise to RES's and other phytoestrogen effects often employ cell culture techniques. A potential limitation of the *in vitro* approach is that the polyphenol concentrations required to elicit effects are often several fold higher than what is observed *in vivo*. However, it is important to note that the free concentrations of RES in medium are likely to be much lower than the calculated levels. The presence of free RES in medium containing fetal calf serum is reduced to 50% of the initial concentration 2h after its addition, and falls to 0 after a 24h incubation period (Jannin et al., 2004). In the HepG2 cell line, a commonly used model for human

hepatocytes, RES uptake is hypothesized to occur by a combination of passive transport and simple diffusion. RES uptake by simple diffusion is nearly two fold lower in the presence of serum in culture (see Delmas and Lin, 2011 for review), suggesting that the presence of serum affects the free concentrations of RES in culture and in turn its cellular uptake.

In an attempt to prevent the interaction between fetal calf serum in culture medium and RES, experiments were conducted using a serum-free medium formulation. In lung fibroblasts (MRC5) RES treatment in serum free conditions actually increased proliferative growth rate, which is the opposite of what is observed when RES is added to serum containing medium. Unfortunately, serum free conditions can affect the expression of ERs, in particular ERalpha (Campbell et al., 2002). The potential change in the ratio of ERalpha to ERbeta greatly complicates the interpretation of phytoestrogen effects in cell lines cultured under serum free conditions. The use of this experimental approach to evaluate the requisite concentration of RES to elicit an induction of MnSOD *in vitro* was not pursued.

The high concentrations of RES and phytoestrogens necessary to elicit biological activities *in vitro* may be reflective of its limited free concentration in serum containing medium. In this thesis the concentration of free RES in culture medium and its association with serum proteins was not evaluated, but this should be a goal of future work.

8.6 Resveratrol *in Vivo*

RES undergoes extensive chemical modification in the intestinal tract and is rapidly metabolized. In humans, plasma levels of RES following a supplemental dose of

25mg dose peak at low micromolar concentrations (Walle et al., 2004). Similarly, in rodents, oral intake of RES in the hundreds of milligrams range also yields plasma levels that are only in the low nanomolar range (Teng et al., 2012; Marier et al., 2002), with the highest levels observed in liver and kidney tissue (Juan et al., 2010). One strategy to overcome RES's very low bioavailability may be to maximize the dosage used. For example, while a single 25mg dose of RES yields low nanomolar concentrations of RES, a single 5g dose increases this to low micromolar concentrations in plasma (Boocock et al., 2007). In this context it is important to note that there have been no reports of serious adverse side effects associated with these high doses of RES in humans (Edwards et al., 2011). Thus, the possibility exists that very high supplemental doses of RES will provide the low micromolar concentrations of RES in plasma that can elicit biological activities *in vitro*.

The identification of MnSOD as a key component of RES's molecular mechanism is corroborated by data showing that MnSOD levels increased in the brain tissue of male mice given RES in a high fat diet (Robb et al., 2008b; Chapter 6). The effect on MnSOD was exclusive to brain tissue, and occurred only when RES was provided in a high fat diet. The reasons for the tissue specificity are unclear, and while it may be reflective of a difference in the abundance of ERs between tissues this idea was not explored in this study. In any case, the ability of RES to elicit an induction of MnSOD in a tissue vulnerable to stress-induced cell death may represent an exciting therapeutic strategy. Indeed, RES treatment protects against neuron loss in mice given the toxin MPTP (a pharmacological model of Parkinson's), concurrent with an upregulation of MnSOD

(Mudo et al., 2012). Interestingly, the neuroprotective effect of RES was subsequently related to its actions at estrogen receptors (Di Liberto et al., 2012).

The ability of RES to elicit many of the beneficial effects of MnSOD overexpression *in vivo* is an exciting observation for this compound for its potential to provide a dietary means of achieving the therapeutic benefits of MnSOD overexpression.

9. Conclusions

Changes in mitochondrial function and structure can affect proliferative cell growth and stress resistance, two important properties in the context of many age-related diseases including neurodegeneration and cardiovascular dysfunction. Data gathered from animal studies and from preliminary human trials support the idea that the phytoestrogen RES has broadly protective effects on human health.

The cellular mechanisms that give rise to these beneficial effects on health continue to be the focus of much research. My data indicate that the ability of RES to inhibit proliferative growth and and confer cytoprotection in fibroblasts and myoblasts is mediated by ERbeta and the downstream induction of MnSOD expression.
This observation suggests that other phytoestrogens with ERbeta agonism activity should elicit similar effects. Indeed, pterostilbene and piceid, two polyphenols that are abundant in red wines, and the phytoestrogens coumestrol, kaempferol, genistein, and daidzein, induce similar increases in MnSOD expression, stress resistance and slowed growth in fibroblasts, myoblasts and human prostate cancer cells.

Interestingly RES's effects on stress resistance and cell growth may be phenocopied with mdivi-1, an inhibitor of mitochondrial fission. Treatment of myoblasts, fibroblasts and prostate cancer cells with RES and the ERbeta agonist DPN stimulates fusion of the mitochondrial reticulum. Given that increased mitochondrial fusion is known to impede cell cycle progression and to increase resistance to a variety of stressors, I propose that this is an important mechanistic connection between RES, ERbeta agonists, MnSOD, and the changes in cell behaviour (Fig 9.1)

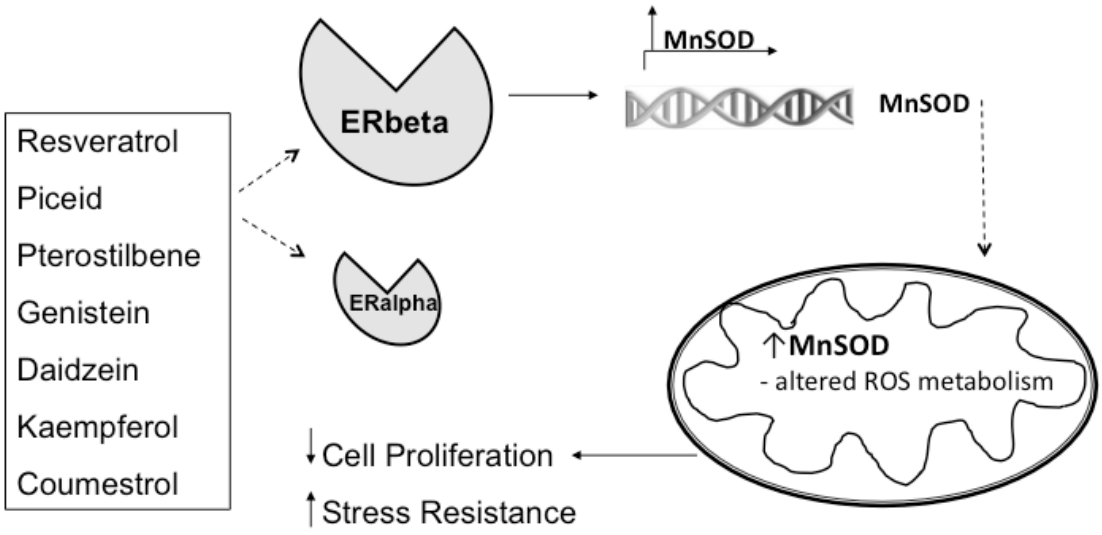


Figure 9.1 Resveratrol and derivatives affect cellular stress resistance and proliferative growth via primarily ERbeta-mediated modulation of mitochondrial ROS metabolism.

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